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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
WOLF-RUEDIGER SCHAEBITZ, ET AL.	: EXAMINER: BORGEEST
SERIAL NO: 10/659,295	:
FILED: SEPTEMBER 11, 2003	: GROUP ART UNIT: 1649
FOR: METHODS OF TREATING NEUROLOGICAL CONDITIONS WITH HEMATOPOIETIC GROWTH FACTORS	:

DECLARATION UNDER 37 C.F.R §1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

Wolfgang Kuschinsky states that:

1. I am a Professor of Physiology at the University of Heidelberg.
2. I have a doctorate in Medicine from University of Munich and understand that the relevant field in question is arteriogenesis and treatments of traumatic brain injury.
3. I have 39 years experience in this field.
4. I have cooperated with Sygnis Bioscience GmbH & Co KG (formerly Axaron) for 8 years in the field of cerebral ischemia.
5. I have read and understand the contents of the above-identified application as well as the current claims to the treatment of traumatic brain injury with G-CSF and/or G-CSF derivatives.
6. I understand that one of the issues the U.S. Patent Office has raised in this application is that the method of treating traumatic brain injury in an individual

with G-CSF or derivatives of G-CSF would have been considered obvious to someone in this field based on what is described in WO 99/17798 (Buschmann et al) and the article of Bouma et al (1992) *J Neurosurgery* (77):360-368).

7. I have read and understand what each of these publications described.
8. It is my view that one would not have combined the knowledge in Bouma et al and WO 99/17798 due to the very different time windows underlying the conditions each sought to treat and indeed would lead one away from treating traumatic brain injury with G-CSF as described in the current application.
9. This conclusion is based on the fact that Bouma et al teach that traumatic brain injury is accompanied by an early and transient ischemic event a few hours after head injury (see Bouma et al p. 366, left column: "*Recently, however, we have found evidence that ischemia ... if present, only occurs in the first few hours after severe head injury, while after 24 hours the mean CBF [, cerebral blood flow,] usually has increased to values suggestive of 'relative hyperemia' ...*" and "*... confirming the hypothesis that posttraumatic ischemia is usually an early event ...*" and "*In our earlier study, the 33 % incidence of ischemia found between 4 and 6 hours postinjury rapidly decreased with time ...*").
10. On the other hand the stimulation of arteriogenesis which is the growth of pre-existing collateral arteries according to WO 99/17798 is a comparably slow process which needs several days up to few weeks to develop. The WO 99/17798 patent application itself reports the enhanced growth of collateral arteries within 7 days (see the description of the femor ligation experiment in Example 1).
11. Said another way, traumatic brain injury induced ischemia as discussed by Bouma et al has already ended when the arteriogenesis according to WO 99/17798 begins.

12. The attached publications, discussed below, provide further basis for the timing at which arteriogenesis begins when compared to traumatic brain injury induced ischemia as described by Bouma et al.

13. Paskins-Hurlburt et al. (1992) *Cir. Res.*70(3):546-553 report that more than 1 week is needed for the growth of collateral arteries (see page 550, left column "... *the collateral arteries were inadequate at 1 week. At that time they would not support muscle work, and basal and peak blood flow were reduced. Skeletal muscle contractile responses and blood flow, however, clearly had returned to normal by 3 weeks after superficial femoral artery ligation.*").

14. Buschmann et al (2001) *Atherosclerosis* 159: 343-356 report the growth of collateral arteries with and without GMCSF administration 7 days after the ligation event. (see abstract: "*The continuous infusion of GM-CSF for 7 days into the proximal stump of the acutely occluded femoral artery of rabbits by osmotic minipump produced indeed a marked arteriogenic response* ...").

15. Herzog et al. (2002) *Am J. Phsiol Heart Cir. Physiol.* 283:H2012-H2020 report the development of collateral arteries within 1 to 2 weeks (see p. H2018, left column: "... *studies on acute coronary occlusions have shown that collateral arteries grow within 1-2 wk in a majority of patients.*").

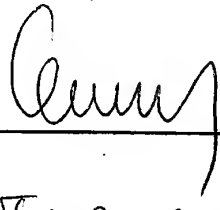
16. Further, arteriogenesis is based on cell division processes. Because of the time needed for such processes it appears impossible to improve blood circulation sufficiently to treat, by the stimulation of arteriogenesis, a transient ischemia occurring only hours after an injury.

17. What this collective information tells me, and also one with relevant knowledge and experience in this field, is that arteriogenic G-CSF would not be a candidate for treating traumatic brain injury. Indeed, I would not consider stimulation of

arteriogenesis to treat a transient ischemia occurring only hours after the injury and therefore not for the treatment of traumatic brain injury as defined by the claims of the application here.

18. That the application describes both a neuroprotective effect and neuroregenerative effect provides a different concept and a real avenue into treating traumatic brain injury. Without these data, presented in the application, I would not have had any reason to try using G-CSF for treating traumatic brain injury nor any expectation that such a regimen would work.

19. The undersigned declares further that all statements made herein are of his own knowledge, are true and that all statements made on information are believed to be true. Further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.



Signature

Feb 25, 2008

Date

Collateral arteries grow from preexisting anastomoses in the rat hindlimb

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AJP - Heart 283:2012-2020, 2002. First published Jul 18, 2002; doi:10.1152/ajpheart.00257.2002

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Collateral arteries grow from preexisting anastomoses in the rat hindlimb

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Herzog, Sandra, Hendrik Sager, Eugen Khmelevski, Andrea Deylig, and Wulf D. Ito. Collateral arteries grow from preexisting anastomoses in the rat hindlimb. *Am J Physiol Heart Circ Physiol* 283: H2012–H2020, 2002. First published July 18, 2002; 10.1152/ajpheart.00257.2002.—Previous findings have suggested that collateral arteries grow from preexisting arteriolar anastomoses ("arteriogenesis"). To investigate whether collateral growth occurs without preceding angiogenesis, we obtained vascular casts and postmortem angiographies 3, 7, and 21 days after unilateral femoral artery occlusion in the rat. Proliferation kinetics were determined after 5′-bromo-2′-desoxyuridin infusion. A preexisting anastomosis was identified. Proliferation of this vessel began 24 h after femoral artery occlusion, increased maximally during the first 3 days, and reached 60% at *day 7*. Cell division was restricted to preexisting anastomoses and occurred neither in directly neighboring arterial vessels nor in capillaries. Collateral vessels doubled their diameter within 7 days and assumed a typical corkscrew appearance (increase of length: 21%). After 7 days of occlusion, we measured a further increase of length (14%) but no proliferation or increase of diameter. We conclude that arteriogenesis is a biphasic process involving rapid proliferation of preexisting arteriolar shunts followed by pronounced remodeling processes. Arteriogenesis occurs independently of angiogenesis and denotes a separate entity of vascular proliferation.

angiogenesis; arteriogenesis; collateral growth; vascular remodeling

MECHANISMS AND STIMULI leading to collateral growth have been subject of intense debate during the past decade (16, 26, 31). The dispute has been difficult to solve because the growth of a complex structure like that of a collateral artery has evaded direct observations. Angiogenesis, the sprouting of capillaries, in contrast, can be studied in in vitro assays, in chick allantois membrane assays, or in implanted tumors via intravital microscopy. Most adaptive mechanisms in the adult are a recapitulation of embryonal developments. In the embryo, a sequence of events has been described leading from vasculogenesis, the in situ accumulation and differentiation of endothelial precursor cells into primary vascular structures, and subsequent angiogenesis, the sprouting of capillaries from preexisting vessels, to the formation of vascular networks. Shortly before birth, this primary network undergoes profound remodeling processes, including invasion of smooth muscle cells as well as regression of other vascular structures that result in the formation of distinct vascular provinces (25). Hypoxia was identified as a major stimulus for angiogenesis during embryonal development as well as during tumor growth (13). Several growth factors and receptors responsible for the different stages of vascular development as well as mechanisms for their induction have been described (31). The most important growth factors originate from the VEGF and FGF family. The angiopoeitins appear to be responsible for the necessary assembly and disassembly of vascular structures, and the ephrins appear to be responsible for growth guidance (4, 5). Little, however, is known about the final remodeling step that leads to formation of a mature vasculature. The notion that collateral growth is a recapitulation of the whole process of vascular development in the embryo or at least of hypoxia-driven angiogenesis, followed by subsequent remodeling processes has been challenged by several observations. One of these observations was that collateral arteries grow largely outside the ischemic area at risk and that growth continues beyond the time of peripheral tissue ischemia (14). Fulton (9) had already demonstrated this for the human heart in 1965 merely by analysis of the vascular architecture (9). We were able to show that larger parts of collateral arteries were growing outside territories with perfusion deficits in a rabbit model of femoral artery occlusion (14). As discussed numerous times, it is difficult to explain why collateral growth occurs just outside the ischemic territory and not within this region if ischemia was the main stimulus. It also was demonstrated that neither the expression of hypoxia-sensitive genes nor the content of ADP, ATP, or AMP changed within the region of collateral growth, indicating that indeed hypoxia is not present in areas in which collateral arteries are growing (7). Another observation challenging the angiogenesis hypothesis was the rapidity at

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which collateral arteries grow. In 1981, Rentrop et al. (22) demonstrated, with the help of consecutive angiographies after myocardial infarction, that 30% of all patients had a recruitable collateral circulation at the time of coronary artery occlusion. In just 2 wk, 90% of all patients developed recruitable collalateral arteries (22). Our own data from animal experiments are on the same line. In the dog heart, collateral arteries develop ~4 wk after placement of an Ameroid constrictor. Because we know that constrictor placement leads to a hemodynamic relevant stenosis within 2 wk, we again obtained a period of 2 wk, which is necessary for a collateral circulation to develop within the dog heart (24). In our rabbit hindlimb model, the main rise in collateral conductance occurred within the first week after femoral artery occlusion (14). Although not quantified, proliferation appeared to be maximal during the first 3 days after femoral artery occlusion (2). It is difficult to imagine that arterial vessels with a length of several centimeters carrying several layers of smooth muscle cells grow within 2 wk from sprouting capillaries. To explain these findings, we therefore developed the hypothesis that these collateral vessels grow from preexisting arteriolar anastomoses. Upon occlusion of the main nurturing vessel, the major part of the blood flow is diverted through these small preexisting vascular connection, which leads to a dramatic increase in flow velocity in these vessels and therefore to a dramatic rise in shear force. This increase in shear force we believed to be the main stimulus for collateral growth. We named this proposed mechanism "recapitulated arteriogenesis." However, to date, it has not been demonstrated beyond a doubt that it is a preexisting anastomosis that gives rise to a collateral artery. Therefore, our concept has often been misinterpreted in the sense that arteriogenesis merely describes remodeling processes that transform angiogenic blood vessels into arterial vessels (16). This interpretation ascribes adult angiogenesis as the primary mechanism responsible for collateral formation.

In this paper, we demonstrate for the first time that collateral growth can occur without preceding adult angiogenesis. We were able to clearly identify a preexisting arteriolar shunt connecting the internal iliac artery to the popliteal artery in the rat hindlimb. Upon femoral artery occlusion, this vessel starts to proliferate within 24 h and becomes a collateral artery. Vessels of similar size and architecture in the direct vicinity of this vessel that were not part of preformed anastomoses did not proliferate. We were thus able to demonstrate at the level of a single vessel that arteriogenesis occurs independently of angiogenesis and denotes a separate entity of adult vascular proliferation. The ability to follow vascular growth in a single vessel allowed us to analysis the proliferation and growth kinetics for collateral vessels and enabled us to study the early events in arteriogenesis preceding vascular proliferation. This is not possible in models in which the vessels bound to proliferate cannot be identified before they start to grow.

MATERIALS AND METHODS

Animal model. The present study was performed according to Section 8 of the German Law for the Protection of Animals. Experiments were performed on male Sprague-Dawley rats weighing 400–500 g. Anesthesia was induced by ether inhalation and continued by intraperitoneal injections of ketamine (100 mg/kg body wt, Atarost) and 2% xylazine (5 mg/kg body wt, Bayer). The right femoral artery was prepared carefully without damaging the vein and nerve and ligated with two sutures (Resolon 5/0, Resorba) ~1 cm apart, the first one just distal to the arteria femoralis profunda. The wounds were closed, and the animal was allowed to recover. Animals designated for proliferation studies were supplied with osmotic minipumps filled with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Sigma). Osmotic minipumps (model 2Ml1, Alzet, 10 ml/h, 7 days) were filled with 62 mg BrdU dissolved in 3 ml of 0.5 M $NaHCO_3$ buffer (pH 9.8) under sterile conditions. They were implanted subcutaneously in the neck region.

Corrosion casting. Corrosion casting was peformed as previously described (11). Rats were anesthesized and anticoagulated (700 units heparin/kg ip). The abdominal cavity was opened, and the aorta was cannulated. Blood was flushed from the organ with saline (37°C at 80–100 mmHg). Resin (Mercox/catalyst, 10/0.3, Ladd Research Industries; Burlington, VT) was infused through the same cannula until the onset of polymerization. The resin-filled tissue was immersed in hot water (50°C) for 1 h to complete resin curing. The tissue was removed by maceration in alternating rinses of 5% KOH and hot water. After the resulting casts were cleaned in distilled water, the arterial tree, including the collateral arteries were carefully stripped from the capillaries and venous tissue for better visualization. Corrosion castings were obtained from five animals at 2 wk and 2 mo after unilateral femoral artery occlusion.

Postmortem angiography. Postmortem angiographies were obtained as previously described (14). Gelatin (12 g, type A, from porcine skin, Sigma) was dissolved in 100 ml of heated distilled water under continuous steering. Afterward, 60 g barium sulfate (Merck) was added. Animals were anesthesized again and anticoagulated, and the aorta was cannulated. Subsequently, the animals were bled and immersed in water warmed to 37°C. After the lower part of the body was rinsed with saline (37°C at 80–100 mmHg), the contrast medium was infused with a pressure of 150–180 mmHg until filling of the distal femoral stump was observed. The animals were immediately placed on crushed ice, and the contrast medium was allowed to harden under continuous pressure. Before the angiographies were obtained, the rats were embedded in gelatin (type A, from porcine skin, Sigma) to fix the animal and to obtain an equilibrated thickness for X-ray penetration. X-ray pictures were taken in a X-ray chamber (model 43855D, Faxitron X-Ray) with the use of single paper-wrapped films (X-OMAT MA 13 × 18 cm, Kodak) exposed to 30 kV for 6 min. Pictures were taken at two different angles to allow for stereoscopic analysis of vessel architecture. Identification and counting of collateral arteries was performed with help of a stereoscope (Topcon; Tokyo, Japan). This allowed three-dimensional identification of the stem, midzone, and reentry regions of collateral vessels. Angiographies were obtained from five animals after 7 days of unilateral occlusion and three animals after 21 days of unilateral femoral artery occlusion.

Determination of diameter, length, and number of collateral arteries. Only vessels clearly identified as collateral vessels by virtue of a stem, midzone, and reentry region were

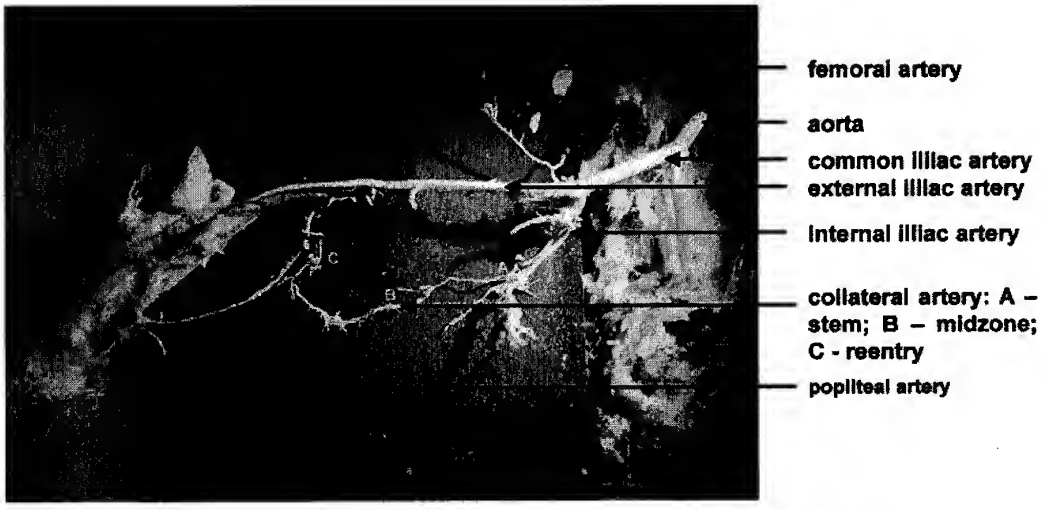

Fig. 1. Corrosion casting of a rat hindlimb preparation without occlusion. A preexisting anastomosis with a stem (*A*), midzone (*B*), and reentry section (*C*) connecting the internal iliac artery to the popliteal artery can be identified.

counted as collateral vessels. Counting was performed by a blinded observer. Collateral vessel diameter and length were obtained from angiographies using NIH Image software. Diameter and length were only determined in the main collateral vessel, which was reproducibly identifiable in all animals studied. For normalization of contrast filling, midzone diameter was calculated as the ratio to the diameter of the femoral artery just distal to the occlusion.

Determination of the proliferative index and proliferation kinetics. After 24 h, 3 days, 7 days, and 21 days, the respective groups of animals were anesthesized again. The aorta was canulated, the animals were bled, and contrast medium was infused as described before. After being hardened on crushed ice, the midzone of the identified collateral vessels was quickly removed, including the surrounding tissue, embedded in Tissue Tek (OCT compound, Sakura Finetek) on cork plates, and shock frozen in *N*-hexane (ICN Biomedicals). Cyostat sections (7 μm thick) were fixed in glycine and ethanol (3:7, pH 2.0) at −20°C over 20 min. Sections were washed in phosphate-buffered saline (PBS) before being blocked with 1% BSA (bovine serum albumin, Fluka) for 30 min. For BrdU staining, we used the BrdU working solution as supplied by the 5-Bromo-2′-Deoxyuridine Labeling and Detection Kit 2 (Roche Diagnostics) according to the protocol supplied by the manufacturer. As a secondary antibody, we used a Cy2-conjugated goat anti-mouse IgG (Lot 41110, Jackson ImmunoResearch Laboratories; Dianova, Germany) diluted 1:200 in 1% BSA. Nuclear staining was obtained using a 0.001% propidium iodide solution (P-4170, Sigma). Before analysis under the fluorescent microscope, slides were embedded in Mowiol (Calbiochem) und 1,2-phenylenediamine (PPD; Merck). For counting purposes, pictures were taken from four to five sections of three different midzone segments of the main collateral vessel. The proliferative index was calculated as the number of BrdU-positive nuclei (green fluorescence) to the total number of nuclei (red propidium iodide fluorescence). The proliferative index was determined in three animals after 24 h, five animals after 7 days, and three animals after 21 days of unilateral femoral artery occlusion and continuous BrdU infusion.

Histology. To evaluate lumen size and vessel wall structure, histological samples of the main collateral artery were obtained after perfusion fixation 3 days, 7 days, 3 wk, and 4 mo after femoral artery occlusion. For perfusion fixation, the aorta was cannulated under general anesthesia and anticoagulation as described above. The lower part of the body was flushed with saline, followed by infusion of 4% formaldehyde at a constant pressure of 150 mmHg for 20 min as described previously (2). After the lower part of the body was flushed again with saline, contrast medium was infused as described above to identify the collateral artery. Midzone segments of the collateral artery were obtained for cryostat sections as described above as well as for paraffin embedding. Cryostat sections were stained with hematoxillin, and paraffin-embedded tissue sections were subjected to hematoxillin-eosin staining.

Statistical analysis. Data are presented as means ± SD. Statistical comparisons between groups were performed with Student's *t*-test. Differences among means were considered significant when $P < 0.05$.

RESULTS

Identification of a preexisting arteriolar anastomose by corrosion casting. Corrosion casts of both lower extremities of Sprague-Dawley rats revealed a preexisting arteriolar shunt connecting the internal iliac artery to the popliteal artery with a defined stem, midzone, and reentry region. The anatomy was reproducible in five experiments (Fig. 1). The vessel had only a slight tortuous appearance. Midzone diameter was 140 μm. After 2 wk and 2 mo of femoral artery occlusion, this vessel became the most prominent collateral artery with an extremely tortuous course and a midzone diameter of 300 μm. Localization and anatomy was the same in all casts examined and was identical for both the dormant anastomosis as well as for the fully developed collateral vessels, indicating that in-

Fig. 2. Postmortem angiogram of a rat hindlimb after 7 days of femoral artery ligation (*A*) and without femoral artery ligation (*B*). A preexisting arteriolar anastomosis connecting the internal iliac artery to the popliteal artery can be identified in the nonoccluded leg (*A*, open arrowheads). This preexisting connection enlarges and becomes a typical corkscrew appearing collateral vessel after femoral artery occlusion (*B*, solid arrowheads).

deed the main collateral artery grows from this preexisting arteriolar anastomosis.

Development of the main collateral artery from preexisting anastomosis demonstrated by postmortem angiography. In the following set of experiments, we created stereoscopic postmortem angiograms before as well as 1 and 3 wk postfemoral artery occlusion for analysis of collateral anatomy and development using computerized imaging systems. Again, the preexisting anastomosis was clearly visible at a reproducible anatomic site in all animals studied and was identified as the vessel becoming the main collateral artery (Fig. 2, *A* and *B*). The total number of angiographically visible collateral vessels increased significantly from 2.3 ± 0.5 before occlusion to 5.3 ± 0.5 after 7 days of occlusion and to 7.3 ± 0.5 after 21 days of occlusion (control vs. 7 days: $P < 0.00001$; 7 vs. 21 days: $P < 0.01$; Fig. 3*A*). The major increase was thus found within the first 7 days after occlusion.

The midzone index (midzone diameter to diameter of the femoral artery distal to the ligature) of the main collateral vessel increased from 0.17 ± 0.05 before

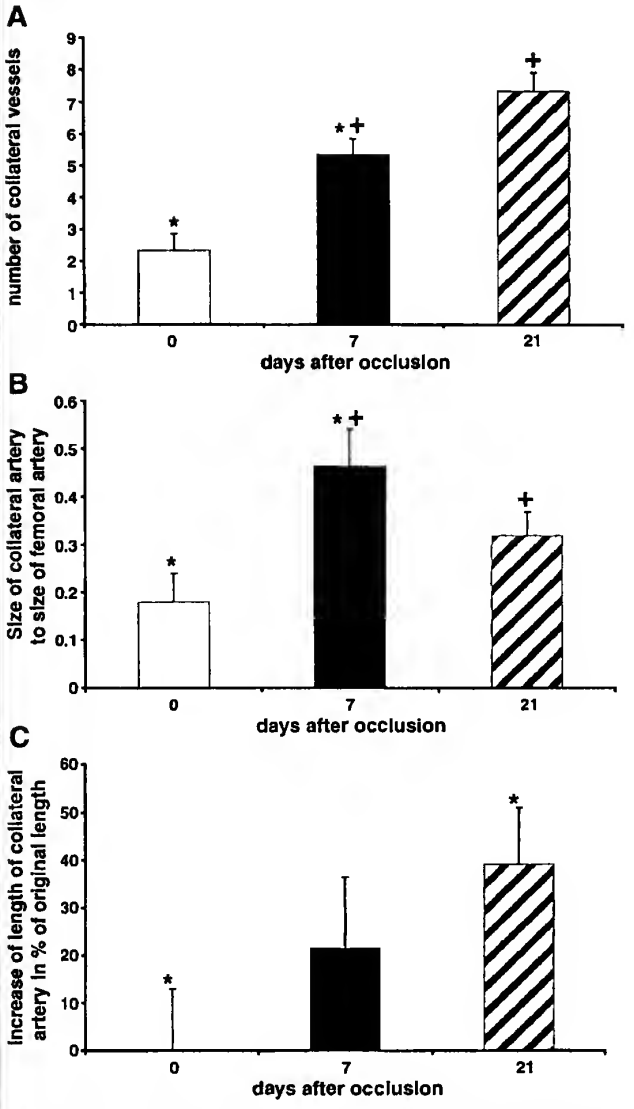

Fig. 3. After femoral artery occlusion, the main increase of the number of angiographically visible collateral vessels is observed during the first 7 days. There is a further but smaller increase in the number of collateral vessels between 7 and 21 days (A; $^*P < 0.00001$ and $+P < 0.01$). The collateral midzone size, expressed as the midzone-to-distal femoral artery ratio, doubles within the first 7 days. There is no further increase but a decrease of collateral lumen diameter between 7 and 21 days after occlusion (B; $^*P < 0.001$ and $+P < 0.02$). The length of the main collateral vessel, expressed as a percentage of the original length of the preexisting anastomose, increases almost constantly until 21 days after occlusion (C; $^*P < 0.02$).

occlusion to 0.46 ± 0.07 after 7 days of occlusion ($P < 0.001$; Fig. 3*B*). Interestingly, the midzone index dropped again to 0.31 ± 0.04 after 21 days of occlusion, indicating regression of the collateral vessel after 7 days of occlusion ($P < 0.02$; Fig. 3*B*).

We observed not only an enlargement in diameter of the collateral vessel but also a distension of the length due to an increase in vessel tortuousity. The total length of the collateral artery increased by 21% within 7 days after occlusion and significantly by 39% within 21 days after occlusion ($P < 0.02$; Fig. 3*C*).

Proliferation is restricted to preexisting arteriolar anastomosis. Staining of the collateral vessel and surrounding structures for BrdU after continuous subcutaneous infusion of the thymidine analog during the first week after femoral artery occlusion revealed that proliferation was restricted to preexisting arteriolar anastomosis (Fig. 4, *A–D*). No proliferation was seen in the directly neighboring vessel of similar size and vessel architecture that did not connect the ischemic to the nonischemic territory as revealed by postmortem angiography before the tissue sections were obtained (Fig. 4, *A* and *E–G*).

Proliferation kinetics of the collateral artery. Proliferation kinetics of the collateral artery were obtained after continuous BrdU infusion for 1, 3, 7, and 21 days. Positively stained nuclei of endothelial and smooth muscle cells represented the total amount of cells proliferating within the observed period and were related to the total amount of nuclei of the vascular tissue. This allowed the calculation of accumulative proliferation indexes. The first BrdU-positive nuclei were seen 3 days after occlusion, indicating that proliferation started between 24 h and 3 days after occlusion. The main rise of the proliferative index occurred between *days 1* and *3* after occlusion reaching 35% at *day 3* (Fig. 5). At *day 7*, the proliferative index had risen to 59%. From the onset of the second week after occlusion, there was no further measurable proliferation. The proliferative index at *day 21* after occlusion was even slightly lower than at *day 7* after occlusion, in keeping with our angiographic data (24 h vs. 3 days: $P < 0.01$; 3 vs. 7 days: $P < 0.01$).

Histology of developing collateral vessels. Hematoxillin-eosin staining of perfusion-fixed tissue revealed pronounced remodeling processes beginning 7 days after occlusion. Remodeling was particularly pronounced between *days 7* and *21* after femoral artery occlusion. Within this time, an asymmetrical neointima was formed including several layers of smooth muscle cells and several laminae elasticae internae (Fig. 6, *A–F*). Within 4 mo after occlusion, vessel wall thickness increased severalfold, whereas lumen diameter of the main collateral vessel in the rat only doubled (Fig. 7, *A* and *B*).

DISCUSSION

In this study, we present for the first time convincing evidence that collateral arteries grow from preexisting arteriolar anastomoses without preceding angiogenesis. We were thus able to deliver proof of the hypothesis that a mechanism distinct from angiogenesis and vasculogenesis is responsible for collateral growth in the rat hindlimb. Our findings are supported by recent observations by Terjung et al. (17), which indicate that collateral growth and angiogenesis respond differently to NO depletion. The question remains as to whether our model is a good reflection of the human disease. As

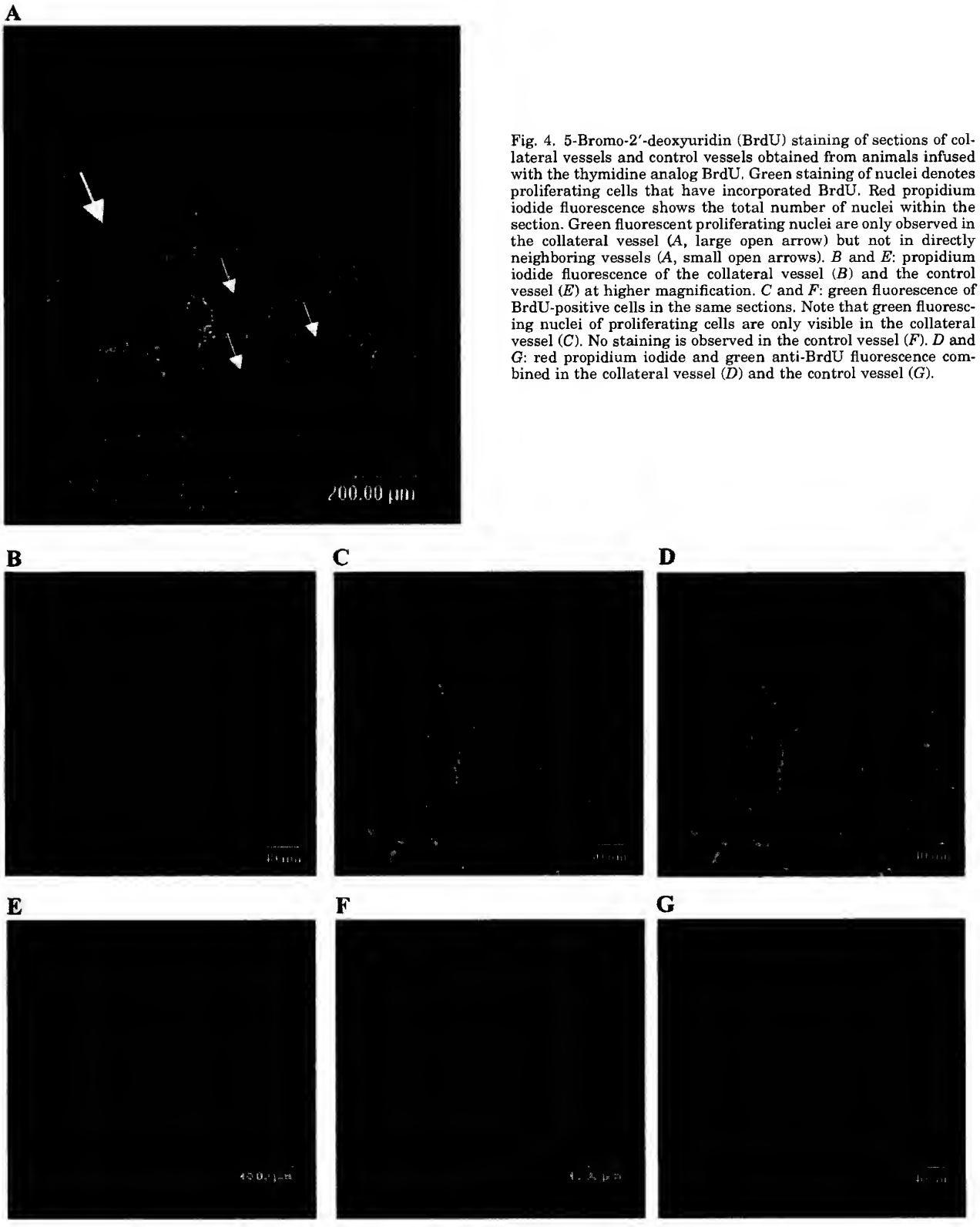

Fig. 4. 5-Bromo-2′-deoxyuridin (BrdU) staining of sections of collateral vessels and control vessels obtained from animals infused with the thymidine analog BrdU. Green staining of nuclei denotes proliferating cells that have incorporated BrdU. Red propidium iodide fluorescence shows the total number of nuclei within the section. Green fluorescent proliferating nuclei are only observed in the collateral vessel (*A*, large open arrow) but not in directly neighboring vessels (*A*, small open arrows). *B* and *E*: propidium iodide fluorescence of the collateral vessel (*B*) and the control vessel (*E*) at higher magnification. *C* and *F*: green fluorescence of BrdU-positive cells in the same sections. Note that green fluorescing nuclei of proliferating cells are only visible in the collateral vessel (*C*). No staining is observed in the control vessel (*F*). *D* and *G*: red propidium iodide and green anti-BrdU fluorescence combined in the collateral vessel (*D*) and the control vessel (*G*).

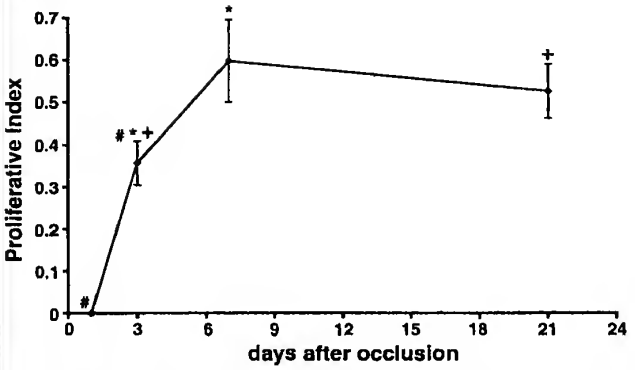

Fig. 5. Proliferation kinetics of the collateral vessel. The main rise in the cumulative proliferative index (numbers of proliferating vascular cells/numbers of total vascular cells) occurs between *days 1* and *3* after femoral artery occlusion. There is a further rise in the cumulative proliferative index between *days 3* and *7*. After *day 7*, no further proliferation is observed (#$P < 0.01$ and *$P < 0.01$; +$P < 0.05$).

mentioned in the Introduction, previous studies have demonstrated the existence of preexisting intra- and intercoronary anastomoses in virtually every human heart and thus the presence of a substrate for the mechanism of collateral growth described in this paper (3, 9). Furthermore, studies on acute coronary occlusions have shown that collateral arteries grow within 1–2 wk in a majority of patients, suggesting that mechanisms similar to those we observed in the rat hindlimb are responsible for vascular growth after myocardial infarction (22). In contrast to acute coronary occlusion, collateral growth takes several months in patients with progressive arterial occlusive disease (21). The stimulus for vascular growth, however, is only present at times in these patients depending on exercise, blood pressure, and several other factors. It usually resolves before severe myocardial damage can occur. The determination of any reliable growth kinetics for such a dynamic situation is nearly impossible. Our study certainly does not preclude a role for angiogenesis under these circumstances. A vascular network would be created de novo giving rise to small arteriolar anastomoses that need to be remodeled to create collateral arteries. At this stage of collateral development, a situation is encountered similar to the one we described for the rat hindlimb.

It was interesting to note that proliferation was restricted to preexisting arteriolar anastomoses but that it did not involve directly neighboring vessels of comparable size and structure that did not connect the nonischemic to the ischemic territory. This confinement of proliferation to certain vessels indicates that the signaling for collateral growth comes from inside the vessel rather than from the surrounding tissues. Thus hemodynamic forces rather than chemical signals released from the surrounding tissue (for example, those evoked by ischemia) are likely to be the primary stimulus for arteriogenesis. Shear force is one of the hemodynamic factors that is altered in a preexisting shunt upon occlusion of the main blood-supplying vessel. Glagov et al. (10) were able to demonstrate in numerous experiments that vessels tend to remodel to maintain a certain level of shear force and tensile force. This general principle may also apply to collateral arteries. In fact, Tuttle et al. (29) were able to show in a recent experiment that the extent of remodeling of collateral vessels in the bowel was proportional to flow velocities and shear forces generated by occlusion of nurturing vessels. Our concept that shear force is the primary stimulus for arteriogenesis was challenged on the grounds that proliferation occurs centripetal from the border of the ischemic zone in the rat kidney (31). Shear force is dependent on flow velocity and the radius to the third power. Thus a centripetal spread of proliferation would be very well explainable by shear force if the radius of the preexisting shunt were smallest at the border of the ischemic organ, which is not unlikely.

Shear force depends on vessels architecture and the kind of flow that is generated. There is a wide variation of flow patterns in corkscrew collateral vessels. Consequently, there will be a diversity of proliferation and remodeling processes along the same collateral vessel if shear force were the main stimulus. This in turn would explain the asymmetry of remodeling processes like neointima formation and the increase in vascular tortuousity.

In this study, we indeed demonstrated an increased in tortuousity reflected in the increase in length of the preexisting collateral vessel. In contrast to proliferation and luminal diameter change, which showed a maximum during the first week after occlusion and then came to a halt, longitudinal distension of the collateral artery continued and reached significant values on *day 21* after femoral artery occlusion. These findings indicate that collateral growth is a biphasic process beginning with a rapid proliferative phase followed by extensive remodeling, which has already been suggested by our previous studies in the rabbit hindlimb (2).

In conclusion, collateral growth is a biphasic process. It begins with a very rapid onset of massive proliferation leading to significant outward remodeling within 1 week after occlusion. This proliferation slows down considerably or even comes to a standstill after 1 wk of occlusion and is succeeded by a phase of intense inward remodeling that leads to neointima formation and a pronounced increase of vessel wall diameter. In the remodeling phase, we even may encounter regression of the collateral lumen diameter, as seen in our model.

Although reasoning based on these findings indicates that not a general chemical factor such as ischemia but local acting hemodynamic forces are responsible for collateral growth, we were not able to deliver direct evidence for the hypothesis that shear force is the primary stimulus for arteriogenesis. The ability, however, to identify a collateral artery at any stage after induction as presented in this paper is the prerequisite for modeling of flow patterns along a preexisting arteriolar shunt and their evolvement during collateral growth.

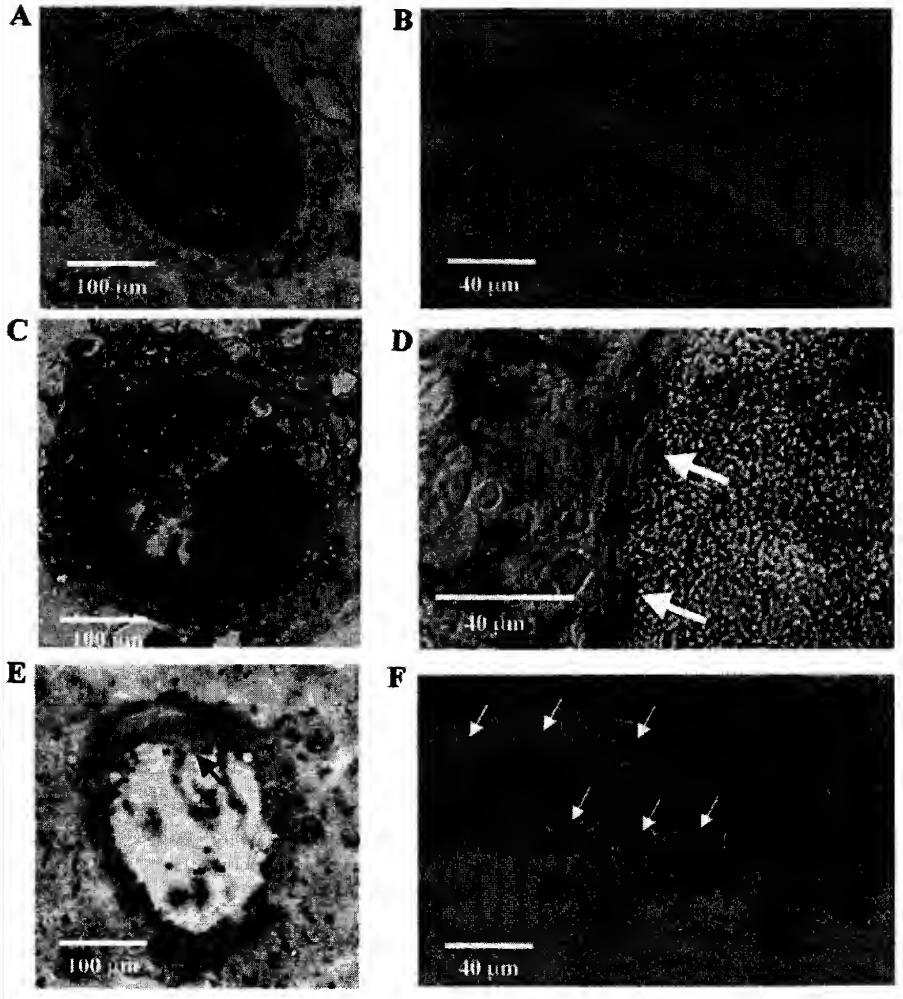

Fig. 6. Cryosections of the collateral artery 3, 7, and 21 days after femoral artery occlusion. The contrast medium-filled vessel is thin walled after 3 days of femoral artery occlusion (*A* and *B*). There is some protrusion of the endothelium in the collateral vessel after 7 days of occlusion (*C* and *D*, large open arrows). After 21 days of occlusion, an asymmetrical neointima (*E* amd *F*, solid arrows) has formed with two laminae elasticae internae (*E* and *F*, open arrows).

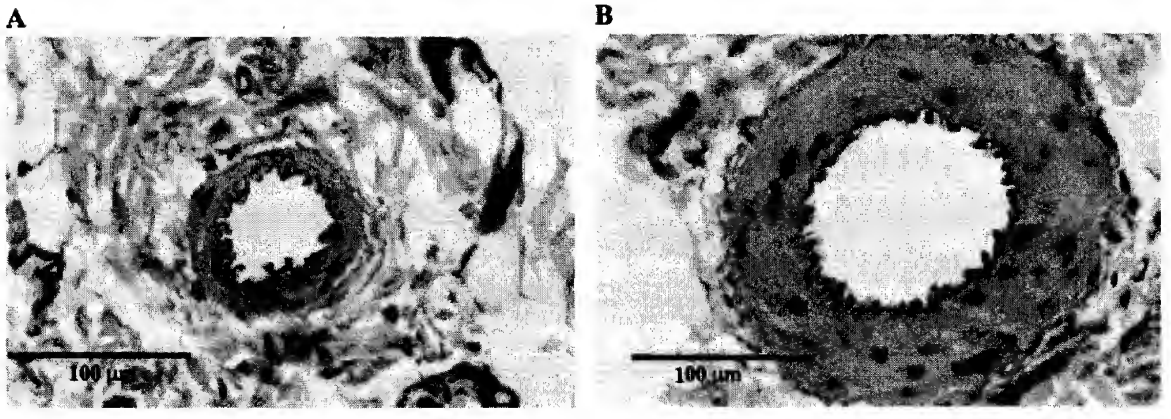

Fig. 7. Sections from paraffin-embedded sections of the perfusion-fixed collateral vessel (*A*) and control vessel (*B*) after 4 mo of femoral artery occlusion. The thickness of the vessel wall has increased severalfold in the growing collateral vessel compared with the same vessel before occlusion, whereas the lumen has only doubled.

The potential molecular base for translating mechanical force into proliferation and remodeling of collateral vessels also remains to be uncovered. Numerous studies, however, have rendered biochemical pathways of how mechanical forces influence cell shape and function (1, 6, 12, 15, 18–20, 23, 27, 30).

The model presented in this paper will allow us to describe the process of arteriogenesis in greater detail. Bearing in mind that all placebo-controlled clinical trials that were based on the concept of inducing angiogenesis to improve vascularisation of ischemic tissue failed to show any significant or lasting effects, the concept of arteriogenesis might aid in finding feasible therapeutical concepts (8, 28).

We thank Juliane Bergmann for continuous technical support and for valuable discussions.

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Time course of arteriogenesis following femoral artery occlusion in the rabbit

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Abstract

Objective: We examined the time course of arteriogenesis (collateral artery growth) after femoral artery ligation and the effect of monocyte chemoattractant protein-1 (MCP-1). **Methods:** New Zealand White rabbits received MCP-1 or phosphate buffered saline (PBS) for a 1-week period, either directly or 3 weeks after femoral artery ligation (non-ischemic model). A control group was studied with intact femoral arteries and another 1 min after acute femoral artery ligation. **Results:** Collateral conductance index significantly increased when MCP-1 treatment started directly after femoral artery ligation (acute occlusion: 0.94±0.19; without occlusion: 168.56±15.99; PBS: 4.10±0.48; MCP-1: 33.96±1.76 ml/min/100 mmHg). However, delayed onset of treatment 3 weeks after ligation and final study of conductance at 4 weeks showed no significant difference against a 4-week control (PBS: 79.08±7.24; MCP-1: 90.03±8.73 ml/min/100 mmHg). In these groups increased conductance indices were accompanied by a decrease in the number of visible collateral vessels (from 18 to 36 identifiable vessels at day 7 to about four at 21 days). **Conclusion:** We conclude that the chemokine MCP-1 markedly accelerated collateral artery growth but did not alter its final extent above that reached spontaneously as a function of time. We show thus for the first time that a narrow time window exists for the responsiveness to the arteriogenic actions of MCP-1, a feature that MCP-1 may share with other growth factors. We show furthermore that the spontaneous adaptation by arteriogenesis stops when only about 50% of the vasodilatory reserve of the arterial bed before occlusion are reached. The superiority of few large arterial collaterals in their ability to conduct large amounts of blood flow per unit of pressure as compared to the angiogenic response where large numbers of small vessels are produced with minimal ability to allow mass transport of bulk flow is stressed.

Keywords: Arteries; Blood flow; Collateral circulation; Macrophages; Microcirculation

1. Introduction

In the adult, blood vessels can grow either via the process of angiogenesis or via the process of arteriogenesis [1–4]. Angiogenesis refers to the sprouting of endothelial cells from pre-existing vessels, thereby forming new capillary networks. One of the main stimulants of this process is hypoxia, increasing the transcription of angiogenic growth factors such as vascular endothelial growth factor (VEGF), which is a known but weak mitogen for endothelial cells [5], via the oxygen dependent regulation of the nuclear protein HIF (hypoxia inducible factor) [6]. Arteriogenesis in contrast occurs independent from ischemia and refers to the proliferation of pre-existing arteriolar connections into functional collateral arteries. When a main artery develops a hemodynamically relevant stenosis, causing a fall in intravascular pressure in the dependent vasculature, blood flow is re-distributed through these interconnecting arterioles, significantly increasing shear stress, which is in turn leading to activation of the endothelium and upregulation of cell adhesion molecules (ICAM, VCAM, selectins) [7–11]. Circulating mononuclear blood cells are attracted by the activated endothelium,

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attach to the CAMs and migrate into the vessel wall, giving rise to the production of various cytokines and growth factors (e.g. bFGF, TNF-α, GM-CSF and MCP-1) [2,9,12,13], finally leading to proliferation and growth of the collateral vasculature. Monocyte chemoattractant protein-1 (MCP-1) has been shown to stimulate the process of arteriogenesis [14] via an increased attraction of circulating monocytes to sites of proliferating collateral arteries.

In comparison to angiogenesis, stimulation of arteriogenesis is probably the more efficient process to replace an occluded artery, because Newtonian flow is related to the fourth power of the vessel's radius, which means that small changes in the diameter of a collateral vessel result in large changes in blood flow. Thus, the increase in blood flow to potentially ischemic tissue, as caused by the development of two or three large collateral arteries, cannot be equaled by newly formed capillaries, however numerous.

One of the most important questions with regard to therapeutic arteriogenesis is that of responsiveness to the agent applied. This first increases but rapidly decreases with time after arterial occlusion. Blood flow measurements in rats showed that 2 weeks after femoral artery ligation the increase in collateral blood flow had stopped. When treated with basic fibroblast growth factor (bFGF), collateral flow increased during the first week about two-fold but did not change significantly in the following weeks [15] although full vasodilatory reserve was not yet reached. These findings and those of Unger [16] and our present results clearly show that therapeutic arteriogenesis has only a limited time-window. Therefore knowledge of the time course of arteriogenesis in any given species is of great importance in order not to miss the responsive cycle of the time window.

Furthermore, weaknesses exist in the experimental evaluation of the time factors as they are influenced by pharmacological agents because of the paucity of sensitive and quantitative methods to study changes as a function of time. Methods developed to provide functional measurements are Laser–Doppler imaging, infrared thermography and oxymetry. In the rabbit hindlimb model these measurements are subjected to considerable method- and operator variability. Furthermore, these techniques measure only derivatives of hindlimb perfusion like flow- or erythrocyte velocity, skin circulation, or skin oxygenation, instead of total hindlimb perfusion.

The only well established and described method to measure directly hindlimb perfusion in vivo after femoral artery occlusion is the use of radioactive tracer microspheres [17,18]. Since their introduction, fluorescent microspheres successfully replaced radioactive tracers in many experimental settings [19–21] and have been shown to be superior in handling, accuracy and reproducibility [22].

In the rabbit hindlimb four different microspheres were used to study the effects of MCP-1 on arteriogenesis in an ex vivo model [14]. In the present study, we measured hindlimb perfusion after femoral artery occlusion as a function of time using six differently labeled fluorescent microspheres at different perfusion pressures at maximal vasodilation. In addition we will describe our experience with a high resolution, low keV X-ray angiography in the rabbit hindlimb model of arteriogenesis.

2. Methods

2.1. Animal model

The present study was performed with the permission of the State of Hessen, Regierungspraesidium Darmstadt, according to Section 8 of the *German Law for the Protection of Animals*. It conforms to the *Guide for the Care and Use of Laboratory Animals* published by The US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Seventy-two New Zealand White Rabbits (NZWR) were randomly assigned to one of six groups ($n=12$ each). After ligation of the femoral artery, two groups received either phosphate buffered saline (PBS) or monocyte chemoattractant protein-1 (MCP-1) (0.2 μg/kg/day) locally via an osmotic minipump for 1 week. To study the long-term development of collateral arteries, animals of groups 3 and 4 were treated with either PBS or MCP-1 for a 1-week period, 3 weeks after ligation. In the fifth group of 12 animals, the femoral artery was ligated directly before the final experiment. To obtain the normal conductance index value and angiographic appearance of the vascular tree of the rabbit hindlimb a sixth group was evaluated without femoral artery ligation. For the implantation of the osmotic minipumps (Model 2 ML 1, Alza Corp., Palo Alto, CA, USA), the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight) and xylazin (8 mg/kg body weight). Supplementary doses of anesthetics (10–20% of the initial dose) were given intravenously as needed. All surgical procedures were performed under sterile conditions. After incision of the skin, the femoral artery was dissected, exposed and cannulated with a sterile polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). The catheter was placed with the tip positioned approximately 1 cm distal to the branches of the arteria circumflexa femoris and the arteria profunda femoris, pointing upstream in order to deliver the substances continuously and during first-pass into the collateral circulation. The incision was sutured carefully to prevent self-mutilation of the animals. Animals received 0.5 ml of intramuscular tetracycline as an antibiotic prophylactic. After the surgical procedure, the animals were housed individually with free access to water and chow and were allowed to move freely. There were no signs of any gross impairment or

necrosis. For final experiments animals of each group were randomly assigned to either angiographic or hemodynamic measurements.

2.2. Post-mortem angiograms

After the treatment period the animals were sacrificed. The distal descending aorta was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm) and briefly perfused with a buffer containing adenosine at a concentration of 1 mg/kg to achieve maximal vasodilation. To prevent premature gelling, all solutions and the animal itself were kept in a water-bath at 37°C. The rinsing procedure was followed by an 8-min infusion with a contrast medium based on bismuth and gelatin [23] at a pressure of 80 mmHg. Subsequently, the contrast medium was allowed to gel by placing the hindlimbs on crushed ice for 60 min. Angiograms of each single hindlimb were taken at two different angles in a Balteau radiography apparatus (Machlett Laboratories) at 30 keV accelerating voltage using a single-enveloped Structurix D7DW film (Agfa). For quantification of visible collateral arteries the resulting angiograms were assessed in a single blinded fashion under stereoscopic viewing in three dimensions. Only vessels indubitably showing a defined stem-, mid- and re-entrant zone according to the Longland classification [24] were counted and marked to make sure that no vessel was counted twice.

2.3. Hemodynamic measurements

For hemodynamic measurements, the animals were again anaesthetized using the same concentrations as listed above. Animals were heparinized with a bolus injection of 5000 Units heparin. The animals were ventilated after tracheotomy and the anesthesia was deepened with a continuous infusion of pentobarbital (12 mg/kg/h). Therefore the jugular vein was dissected and cannulated with a polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). For installation of a pump-driven shunt, the carotid artery was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm). The arteria saphena magna which corresponds to the anterior tibial artery in humans and is the main arterial supply to the lower limb and foot in the rabbit, was exposed just above the ankle and cannulated with a polyethylene catheter (inner diameter: 0.58 mm; outer diameter: 0.96 mm). For sampling of the microsphere reference, the left femoral artery was exposed and cannulated with a polyethylene catheter (inner diameter: 1 mm; outer diameter: 1.5 mm). The distal abdominal aorta was cannulated (inner diameter: 2.2 mm; outer diameter: 2.5 mm) and a pump-driven shunt between the carotid artery and the aorta was installed to perfuse both hindlimbs. Catheters of the saphenous arteries and the cannula of the aorta were connected to Statham P32DC pressure transducers (Statham, Spectramed). A cannulating ultra-sound flow-probe was installed to measure total flow to both hindlimbs. Pressures and total flow were continuously recorded on a computerized recordings system (MacLab, MacIntosh) from which they were later retrieved for further analysis. To achieve maximum vasodilation adenosine (Sigma Chemical Company, St. Louis, MO) was continuously infused (1 mg/kg/min) into the shunt system.

2.4. In vivo pressure–flow relations

After stabilization of peripheral and systemic pressures both legs were perfused at six different systemic pressure levels between 45 and 75 mmHg, using a roller pump (Stoeckert) installed in the above-mentioned shunt between carotid artery and abdominal aorta for maintenance of stable flow levels between 53 and 110 ml/min (Fig. 1). At each resulting different pressure level, differently labeled fluorescent microspheres (either scarlet, crimson, red, blue-green, orange, yellow-green; diameter: 15 μm; Molecular Probes, Eugene, Oregon, USA) were injected into a mixing

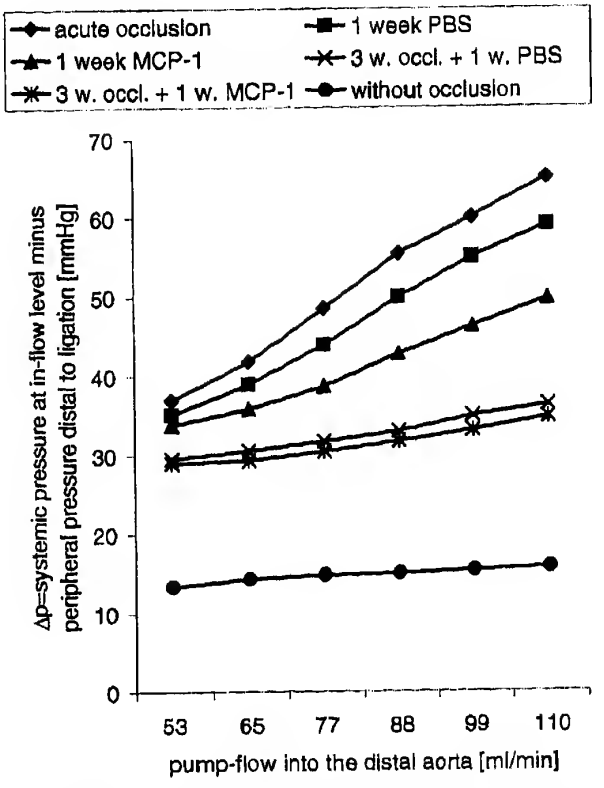

Fig. 1. Relationship between pump-controlled blood flow into the distal abdominal aorta and pressure differences between systemic pressure at the level of in-flow and peripheral pressures distal to the ligation of the femoral artery after different treatment regimens. It is shown that the more efficient the treatment and the higher the conductance index, the lower the pressure difference at the lowest pump-flow and the less steep the curve.

chamber installed in the shunt system. From the left femoral artery a blood sample was withdrawn for 3 min at a rate of 0.6 ml/min as a flow reference for each single microsphere.

2.5. Counting of microspheres[2]

The following muscles were dissected from the leg: quadriceps, adductor longus, adductor magnus, gastrocnemius, soleus, plantaris and peroneal muscles. Each muscle was divided into three consecutive samples (~0.5 g) from the proximal to the distal end. The whole muscle and afterwards each muscle sample were weighted. The samples were then homogenized and placed loosely in 12×75 mm tubes (Becton Dickinson, Lincoln Park, NJ). To each of the tissue samples and to the blood flow reference samples the following was added: 3 ml of a proteinase/SDS solution [SDS stock solution: 1% SDS, 0.5% sodium azide (both Sigma Chemical Company, St. Louis, MO) and 0.8% Tween-80 (Fisher Scientific, Fairlawn, NJ) in 50 mM pH 8 Tris buffer (Sigma Chemical Company, St. Louis, MO)] and 1 mg/ml proteinase K (Boehringer Mannheim Corp.). Blue microspheres (4000/ml, diameter: 15 μm; Molecular Probes, Eugene, Oregon, USA) were used as an internal standard. Each tube was capped and secured in a shaking water bath at 50°C for 24 h. All samples were then centrifuged at 1000×*g* for 30 min, the supernatant was pipetted off and the pellet was resuspended in 1 ml CellWash (Becton Dickinson, Lincoln Park, NJ). Directly before FACS analysis the probes were rigorously shaken. For microsphere counting, a flow-cytometer (FACSCalibur) equipped with a second laser and a detector for a fourth fluorescence was used. After FACS analysis each single microsphere was classified and counted with a computerized analysis system (Becton Dickinson, Lincoln Park, NJ). Flow for each sample was calculated from the number of microspheres in the sample (m_s), the respective microsphere count in the reference sample (m_{rs}), the internal standard microsphere count in the sample (IS_s) and in the reference sample (IS_{rs}), the weight of the reference sample (w) and the time during which the reference sample was withdrawn (t) using the following equation:

$$\text{Flow (ml/min)} = \frac{m_s \cdot IS_{rs} \cdot w}{IS_s \cdot m_{rs} \cdot t}$$

The left hindlimb was processed in the same manner as described above and served as an internal validation group.

[2]The method of using fluorescent microspheres that were counted after tissue digestion in a FACS cytometer was developed by Dr Bruce Ito who shared his experience with us but did not publish it. The description of this method is done with his consent.

2.6. Calculation of conductance indices

In our model, collateral arteries span from the arteria circumflexa femoris and the arteria profunda femoris to the arteria genualis and the arteria saphena parva. After femoral artery occlusion, these vessels provide the blood supply to the lower limb and the distal part of the adductor. Systemic pressures (SP) at the distal part of the abdominal aorta and peripheral pressures (PP) in the saphenous artery were measured. PP is the pressure in the re-entrant region and is identical to the pressure head of the circulation in the lower leg. Collateral flow is equal to the sum of blood flows to the tissues of the distal adductor muscle plus the flow to the tissue of the lower leg. Resistance of the collateral artery network was defined as the pressure difference between SP and PP divided by the collateral blood flow. Conductance is defined as the reciprocal value of vascular resistance and is a recognized parameter in vascular physiology. Because even after maximal vasodilation a positive pressure intercept is observable, all conductance indices were calculated from the slope of the pressure–flow relations.

2.7. Statistical analysis

Results are presented as means±standard deviation. Comparisons between two means were performed using the unpaired Student's *t*-test. Analysis was performed using a computerized software package (Sigma Stat, Jandel Scientific). $P<0.05$ was considered to be statistically significant.

3. Results

No animals were lost during or after femoral artery ligation. We also did not observe any gangrene or gross impairment of hindlimb function after femoral artery occlusion. The body weights and body temperature within the different groups did not show any significant difference. There were no detectable differences in the plasma values of total protein, albumin, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase.

3.1. Angiographic findings

Fig. 2a shows an angiogram of a rabbit hindlimb without femoral artery occlusion. Post-mortem angiograms immediately after occlusion of the femoral artery showed pre-existing interconnecting arteries spanning from the arteria profunda femoris and the arteria circumflexa femoris to the arteria genualis and the arteria saphena parva. These vessels did not show any corkscrew-like formation characteristic for collateral arteries (Fig. 2b). The ligation of the femoral artery led to a proliferation and growth of these pre-existing arterioles. One week after

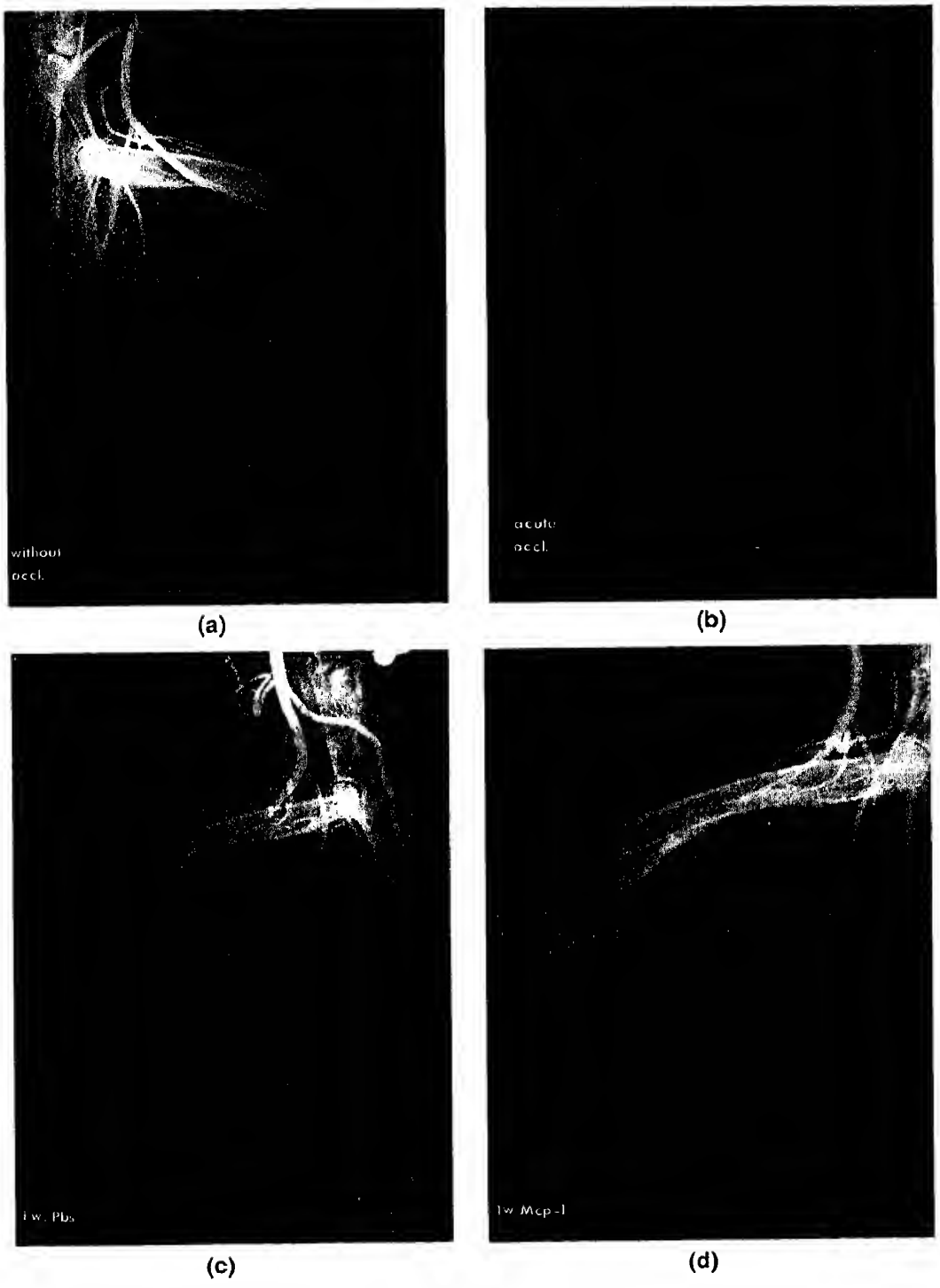

Fig. 2. Postmortem angiograms of rabbit hindlimbs without (a) and after acute femoral artery ligation (b). One week after femoral artery ligation, several collateral arteries spanning the occlusion site can be detected (c). Continuous infusion of MCP-1 for the same time period significantly increases collateral vessel density (d). Animals treated for a 1-week period 3 weeks after femoral artery occlusion showed no significant difference between PBS (e) and MCP-1 (f) infusion.

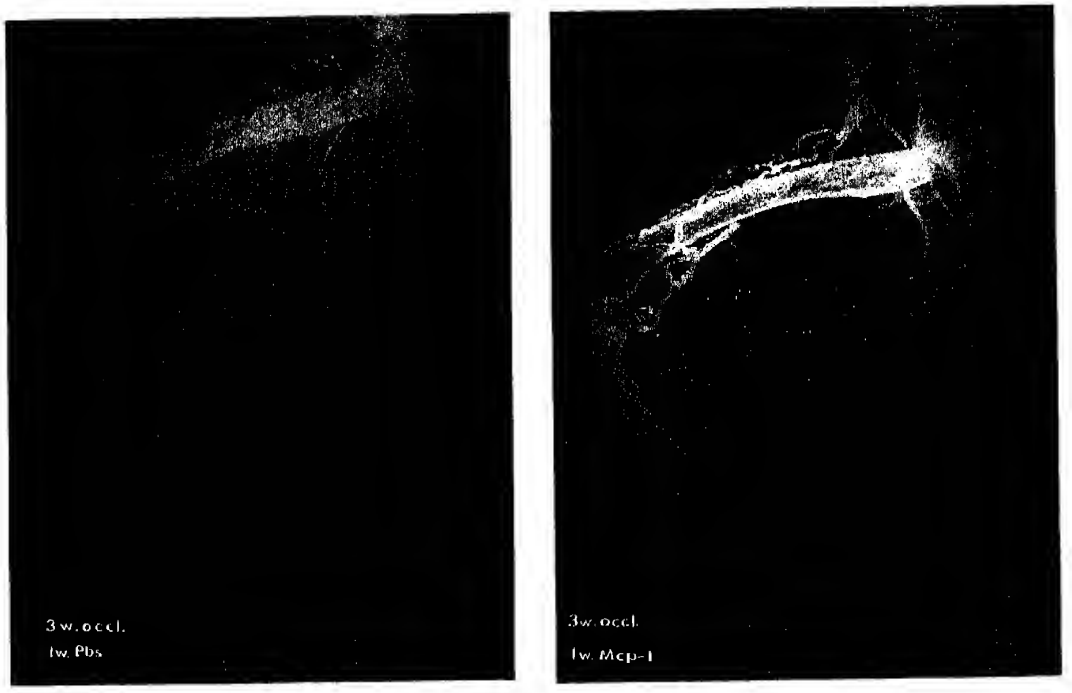

Fig. 2. (*continued*)

PBS infusion the number of visible collateral arteries significantly increased, showing the typical corkscrew formation (Fig. 2c). MCP-1 treatment for the same time period further increased the collateral vessel count (Fig. 2d). The high amount of relatively small collateral arteries declined during the remodeling process resulting in a lower number of vessels with a relatively large diameter both in the control group as well as in the MCP-1 treated group (Fig. 2e and f). Quantification of visible collateral arteries under stereoscopic viewing verified the radiographic appearance (number of angiographically visible collateral arteries: without occlusion: 6.66±1.17; after acute occlusion: 8.27±1.12; 1 week PBS: 16.16±1.40; 1 week MCP-1: 30.16±1.96; 3 weeks occlusion plus 1 week PBS: 10.15±0.98; 3 weeks occlusion plus 1 week MCP-1: 11.07±0.87) (Fig. 3).

3.2. Haemodynamic parameters

The normal conductance index of the arterial vessel bed in the non-occluded rabbit hindlimb was 168.56±15.99 ml/min/100 mmHg. One week after femoral artery ligation the collateral conductance index increased about 4-fold in comparison to the acute occlusion (0.94±0.19 ml/min/100 mmHg; 1 week PBS: 4.10±0.48 ml/min/100 mmHg). MCP-1, given intraarterially as a continuous infusion, significantly increased the collateral conductance index as compared to the PBS-treated group (1 week MCP-1: 33.96±1.76 ml/min/100 mmHg). Four weeks after ligation blood flow was further restored towards normal (3 weeks occlusion plus 1 week PBS: 79.08±7.24 ml/min/100 mmHg). At this time point the collateral conductance index of the MCP-1 treated group did not differ significantly from the control group (3 weeks occlusion plus 1 week MCP-1: 90.03±8,73 ml/min/100 mmHg) (Fig. 4). Conductance index measurements of the left leg, acutely occluded by the catheter from which the reference sample was withdrawn, showed no significant differences between any group (left acute occlusion

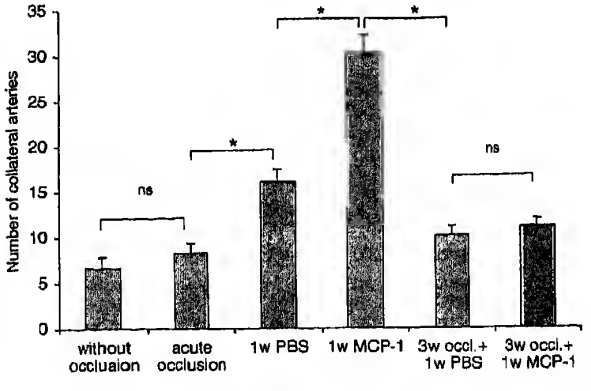

Fig. 3. Number of detectable collateral arteries (* $P<0.05$) counted under stereoscopic view.

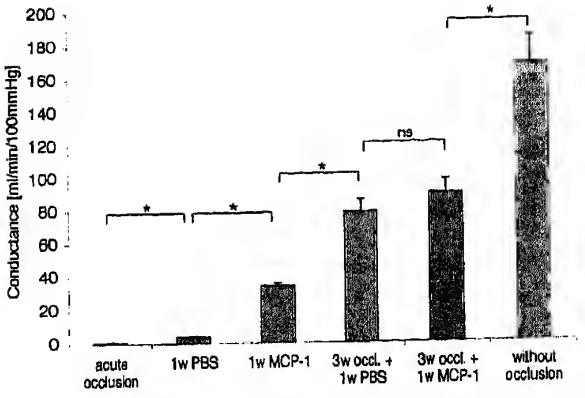

Fig. 4. In vivo conductance indices in ml/min/100 mmHg (* $P<0.05$).

0.91±0.2 ml/min/100 mmHg; left 1 week PBS 0.90±0.11 ml/min/100 mmHg; left 1 week MCP-1 0.96±0.17 ml/min/100 mmHg; left 3 weeks occlusion plus 1 week PBS 0.88±0.17 ml/min/100 mmHg; left 3 weeks occlusion plus 1 week MCP-1 0.91±0.14 ml/min/100 mmHg; left without occlusion 0.88±0.19 ml/min/100 mmHg). Fig. 1 shows the relationship between total flow to the hindlimbs and the difference between systemic and peripheral pressures after different treatment regimens. Low conductance indices correlate with steep curves and vice versa, indicating the maximal capacity of the collateral arteries.

4. Discussion

Although arteriogenesis and angiogenesis are two different mechanisms of vessel growth, exploiting different molecular pathways, they can occur simultaneously, e.g. in the heart. Thus, to study specifically the factors involved in the process of arteriogenesis, without the presence of interfering angiogenic processes, a non-ischemic arterial occlusion model is needed. For that, the rabbit hindlimb model of femoral artery occlusion is an excellent model, since there is no ischemia detectable at rest (no change in e.g. ADP, AMP or lactate levels). Collateral arteries develop in a non-ischemic environment [3] (E. Deindl, unpublished data).

Since the region of interest is non-ischemic, angiogenesis does not significantly contribute to the collateral conductance index measurements. Therefore, this model can be used to specifically examine the process of arteriogenesis and the role of different exogenously applied substances, such as growth factors or cytokines and the molecular and cellular mechanisms responsible for collateral artery growth.

For reliable evaluation of the arteriogenic potency of different substances a reproducible and precise method is required. In this article, we have described an alternative model of tissue perfusion measurements in the rabbit hindlimb using fluorescent microspheres in-vivo. In contrast to the measurement of only one collateral blood flow value, collateral conductance indices were calculated from blood flows and corresponding blood pressures at six different pressure levels between 45 and 75 mmHg. Thereby not only information is obtained about collateral blood flow at a specific pressure but also about the ability of collateral arteries to conduct blood to dependent regions. We analyzed each single sample after specific tissue digestion with proteinase leaving the microspheres unaffected which were subsequently counted for fluorescence intensity by FACS analysis. Each microsphere was classified using its specific spectrum in all four identifiable fluorescence ranges, allowing the use of as many as seven different labels for tissue perfusion measurements. The total number of the different microspheres per measured sample was between 200 and 1000. This number has been shown to allow precise flow quantification [25]. The data provided by the measurements with the fluorescent microspheres are true functional data. However, the unit of conductance (ml/min/mmHg) as used in our model cannot be extrapolated directly to other models since it is specific for the rabbit hindlimb and experimental conditions. We therefore think it is more appropriate to use percentages of normal conductance as shown in Fig. 5.

4.1. Time course of arteriogenesis

Our main finding is that collateral artery growth proceeds in two phases: an early phase with recruitment of numerous pre-existent arterioles which significantly increase conductance within 7 days and a sub-acute phase where conductance rises more markedly in the subsequent 3 weeks because of the selective growth of a few large caliber vessels to the disadvantage of numerous small ones that regress again by a process called 'pruning'. These late large collateral vessels show a 10-fold increase in diameter as compared to the pre-existing arteriolar connection from which they developed. Fig. 6a shows the relationship comparing PBS treatment and MCP-1 treatment, indicating that MCP-1 at first accelerates the process of arteriogenesis. However, at later time-points both curves come together again and will most likely have the same end-point. As shown in Fig. 6b an inverse relationship is observed between the number of collateral vessels and the conductance index of the collateral circulation. This might seem paradoxical, however it reflects the remodeling of the hindlimb circulation where small vessels are 'pruned' away to the advantage of the larger few that conduct blood more efficiently with lesser energy losses, indicating the advantage of arteriogenesis over angiogenesis for flow restoration after arterial occlusion.

It is of note that even in the young healthy animals of our study maximal dilatory reserve is restored to only 50% of the value before occlusion. Four weeks after ligation, collateral conductance index had increased to 47% of the

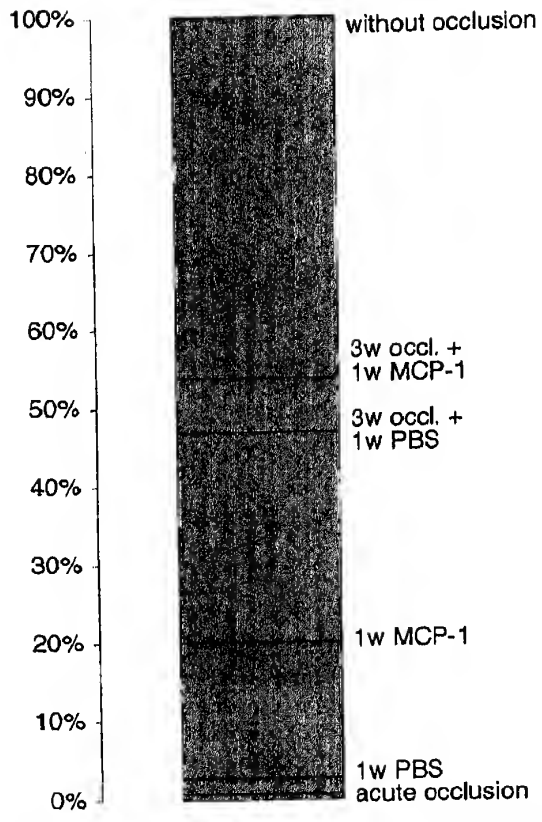

Fig. 5. Conductance percentages compared to normal conductance without femoral artery ligation (=100%). There is a remarkable increase from the situation acutely after femoral artery occlusion and the situation 4 weeks later showing the possible impact of the collateral circulation.

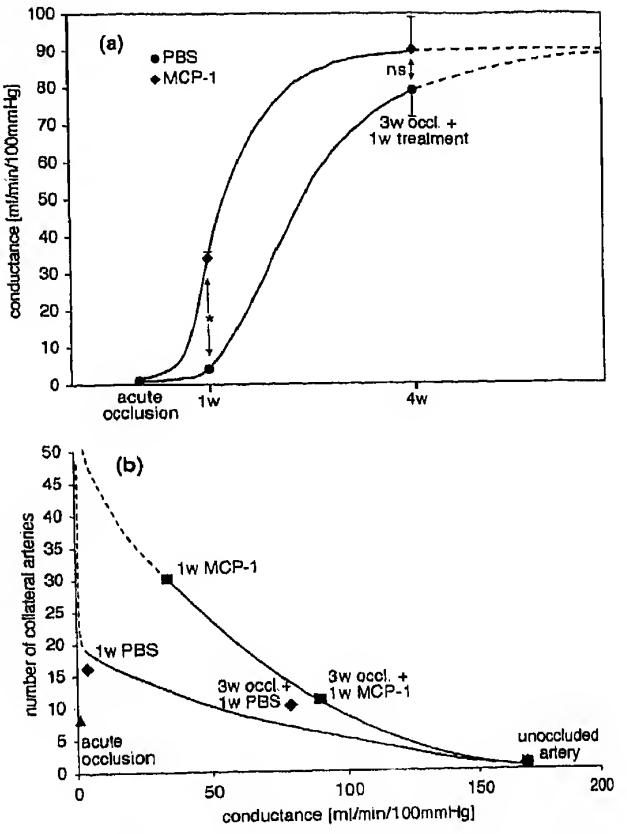

Fig. 6. (a) Increase in collateral conductance index in time after femoral artery ligation for MCP-1 treated and control animals (ns = not significant, * $P<0.05$). (b) Relationship between collateral conductance index (x-axis) and number of detectable collateral arteries (y-axis). After acute occlusion only very few collateral vessels are angiographically detectable; however, the true number is much higher. After 1 week, the number of collateral arteries as well as the collateral conductance index has increased significantly. Four weeks after occlusion of the femoral artery collateral conductance index showed a further increase, whereas the number of collateral arteries has decreased. The correlation between the unoccluded femoral artery and its conductance index shows that numerous smaller vessels cannot simply replace a large artery.

physiologic values, which is almost 20-fold higher than 1 week after ligation and about 80 times as high as after acute occlusion. However, it is intuitively clear that the result in atherosclerotic patients with multiple risk factors is probably less favorable. Although we show here that the time course of collateral development can be significantly accelerated by infusion of the chemokine MCP-1, the final conductance index value reached under the influence of the chemokine is not superior to the spontaneous development if sufficient time was allowed to elapse. Once the spontaneous development had come to an end, a late treatment with the arteriogenic chemokine had no significant effect. The ultimate goal of all further experimental efforts will be the restoration 'ad integrum' by collateral arteries of the maximal conductance of the artery before occlusion.

4.2. Clinical relevance

In case of chronic arterial occlusion, the human body is capable to build own natural bypasses by collateral artery growth (arteriogenesis). However, due to largely unknown mechanisms the process of arteriogenesis usually falls short of complete restoration of maximal conductance giving rise to clinically observable limitations of organ function, especially under loading conditions. This group of patients will most probably benefit from the stimulation of arteriogenesis and some promising results with substances promoting arteriogenesis in an experimental setting were published over the last few years [14,15,26]. However, our data indicate that growth factor treatment may only be effective during a relatively narrow time-window as was already suggested by reports on coronary collateral arteries in the dog and femoral collateral arteries in the rat [15,16]. It remains to be demonstrated if the gain in time is of sufficient relevance in a clinical setting where most often the time of occlusion is either not known or has occurred some time ago.

5. Conclusion

The development of collateral conductance over time shows that maximal conductance values, as in non-occluded femoral arteries at maximal vasodilation, are not reached by growth of pre-existing collateral arteries within the time frame of four weeks. Within that time only 47% of normal maximal blood flow is restored, compared to 1% acutely after ligation. The exogenous application of MCP-1 acutely after femoral artery ligation in the rabbit can significantly accelerate the process of collateral artery formation reaching the final outcome several weeks earlier, probably meaning an important gain of time in the treatment of occlusive artery disease. However, when applied sub-acutely (3 weeks after occlusion) no further positive effect was observed indicating a narrow time-window for growth factor responsiveness, which might be prolonged by combination of different factors.

Acknowledgements

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GM-CSF: a strong arteriogenic factor acting by amplification of monocyte function☆

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Abstract

We investigated the role of the colony stimulating factor for monocytes (GM-CSF) to test the hypothesis whether prolongation of the monocyte's life cycle will support arteriogenesis (rapid growth of preexisting collateral arteries). This appeared logical in view of our discovery that circulating monocytes play an important part in the positive remodeling of small preexisting arterioles into arteries to compensate for arterial occlusions (arteriogenesis) and especially following our findings that MCP-1 markedly increases the speed of arteriogenesis. The continuous infusion of GM-CSF for 7 days into the proximal stump of the acutely occluded femoral artery of rabbits by osmotic minipump produced indeed a marked arteriogenic response as demonstrated by an increase (2-fold) in number and size of collateral arteries on postmortem angiograms and by the increase of maximal blood flow during vasodilation measured in vivo by blood pump perfusion of the hindquarter (5-fold). When GM-CSF and MCP-1 were simultaneously infused the effects on arteriogenesis were additive on angiograms as well as on conductance. GM-CSF was also able to widen the time window of MCP-1 activity: MCP-1 treatment alone was ineffective when given after the third week following occlusion. When administered together with GM-CSF about 80% of normal maximal conductance of the artery that was replaced by collaterals were achieved, a result that was not reached before by any other experimental treatment. Experiments with cells isolated from treated animals showed that monocyte apoptosis was markedly reduced. In addition we hypothesize that GM-CSF may aid in releasing pluripotent monocyte (stem-) cells from the bone marrow into the circulation. In contrast to MCP-1, GM-CSF showed no activity on monocyte transmigration through- and also no influence on monocyte adhesion to cultured endothelial cells. In conclusion we have discovered a new function of the hemopoietic stem cell factor GM-CSF, which is also a powerful arteriogenic peptide that acts via prolongation of the life cycle of monocytes/macrophages.

Keywords: Chronic ischemic heart disease; Collateral arteries; Monocytes; Lipid and lipoprotein metabolism; Atherosclerosis; Colony stimulating factors

1. Introduction

Circulating monocytes play a central role in both innate and acquired immunity of the host. Besides their crucial role in the defense against invading pathogens and a variety of other functions, monocytes play an obligatory role in adaptive growth and tissue remodeling of newly recruited collateral arteries (arteriogenesis) [1–8]. However, the acquisition of functional compe-

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tence and the ability to respond to a variety of activating or modulating signals require maturation and differentiation of circulating monocytes to macrophages, which in turn undergo several biochemical and phenotypic changes [9]. Importantly the process of monocyte activation also confers survival signals essential for the functional integrity of these cells. Under the influence of chemoattractants such as TNF-α and MCP-1 patrolling monocytes can be effectively recruited from the circulation to local inflammatory sites by attachment to the endothelium [10,11]. Normally these monocytes spontaneously undergo programmed cell death unless given 'permission' to survive by special growth factor [12,13]. This indicates that apoptosis may function as a major mechanism for reducing acute inflammation by elimination of unwanted cellular responses. During transendothelial migration endothelial fas-ligand (fas-L) is one of the first important regulators that inhibits monocyte extravasation already during rolling and adhesion by inducing apoptosis via the monocytic Fas-receptor [14–17]. Circulating monocytes have a relatively short life span [18] and their numbers are maintained by continuous replenishment from hemopoietic stem cells, which in turn are dependent on the presence of a variety of accessory stromal cells including fibroblasts, macrophages, adipocytes and endothelial cells in the bone marrow. Together with some of the mature blood cells, these cells are responsible for the production and presentation of a complex array of biologically active proteins (in particular colony-stimulating-factors (CSFs)) which influence the development of blood cells. Granulocyte-macrophage CSF (GM-CSF) enhances the survival [19], proliferation [20] and rate of differentiation [21] of separate hemopoietic cell populations. Therefore, these substances are clinically used to treat patients with hematologic and oncologic disorders [22,23]. Furthermore, several studies have demonstrated that CSFs influence lipid metabolism. They lower plasma cholesterol levels in humans, primates and hypercholesterolemic rabbits by enhancing the clearance of LDL through both LDL-receptor-dependent and independent pathways [24–26]. In addition atheromatous lesions in the aortic arch of Watanabe heritable hyperlipidemic rabbits (WHHL) treated with human macrophage-CSF (M-CSF) were significantly reduced as compared with normal control rabbits, indicating that CSFs prevented the progression of atherosclerosis [24,26–31].

We have previously shown that adhesion, activation, and migration of monocytes play an important role in collateral artery growth [1,4–6]. After ligation of the arteria femoralis in the rabbit, shear forces in preexisting collateral arteries increase significantly, which leads to the upregulation of cell adhesion molecules (e.g. ICAM-1) [2] and endothelial cytokine production such as MCP-1 and GM-CSF. These factors combine to effectively recruit circulating cells, in particular monocytes, to the sites of collateral artery growth. The monocytes itself mature into macrophages, which produce large amounts of growth factors (MCP-1, b-FGF [1]) as well as degratory enzymes such as metalloproteinases [32]. These factors in turn create an inflammatory environment, which is necessary to build an artery from an arteriole. Increased activation and attraction of circulating monocytes via intra-arterial infusion of MCP-1 into the collateral circulation can significantly influence this process of adaptive muscular collateral artery growth (arteriogenesis) [5]. In contrast, functional blockage of ICAM-1 dependent adhesion and transmigration of circulating cells (e.g. monocytes) via infusion of monoclonal antibodies against ICAM-1 in vivo significantly reduces collateral artery growth, thereby supporting the hypothesis that circulating cells dependent on ICAM-1 for transmigration are obligatory mediators of the arterial changes seen with MCP-1 [7].

Since GM-CSF is antiatherogenic and a powerful factor that reduces monocyte apoptosis and is upregulated in endothelial cells under increasing shear stress [33], we have now tested the hypothesis that locally delivered GM-CSF is capable of promoting collateral artery growth by a direct effect on the rate of apoptosis of circulating and adhering monocytes as well as newly-recruited macrophages.

2. Material and methods

2.1. Cytokine

Recombinant human GM-CSF synthesized by *Escherichia coli* was purchased from Novartis Pharma GmbH (Nürnberg, Germany).

2.2. Animal model

The present study was performed with the permission of the State of Hessen, Regierungspraesidium Darmstadt, according to section 8 of the *German Law for the Protection of Animals*. It conforms with the *Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health* (NIH Publication No. 85-23, revised 1985).

2.2.1. Group 1

One week intraarterial cytokine treatment following femoral artery occlusion: for hemodynamic measurements four groups (each $n = 6$) of NZWR received following infusions into the collateral circulation,

GM-CSF (100 μg/day;10 μl/h); MCP-1 (0.5 μg/day), the combination of both or solvent.

2.2.2. Group 2

(One week intraarterial cytokine treatment 3 weeks after occlusion) 24 NZWR were subjected to 21 days of right femoral artery occlusion. At this time the collateralization of these animals was completed before a growth factor treatment was started. After 21 days the animals were randomly assigned to identical treatments like in group 1. Seven or 21 days after pump implantation hemodynamic parameters were obtained. For the initial implantation of the osmotic minipumps, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (40–80 mg/kg body weight) and xylazine (8–9 mg/kg body weight). Supplementary doses of anesthetic (10–20% of the initial dose) were given intravenously as needed. The surgical procedure was performed under sterile conditions. Femoral arteries were exposed and cannulated with a sterile polyethylene catheter (inner diameter, 1 mm; outer diameter, 1.5 mm) pointing upstream, with the tip of the catheter positioned distal to the branching of the arteria circumflexa femoris and the femoral artery distal to the catheter insertion site was ligated. The catheter was connected to an osmotic minipump (2ML-1, Alza Corporation, Palo Alto, CA), which was implanted under the skin of the lower right abdomen. The absence of any residual pump volume (< 3%) after the experiment verified delivery of the contents. After closure of the incision and subcutaneous application of antibiotics, the animals were outfitted with plastic collars that allowed them to move freely but prevented self-mutilation. The rabbits were housed individually with free access to water and chow to secure mobility. Seven days after implantation the animals were again anesthetized with an intramuscular injection of ketamine hydrochloride and xylazine for tracheostomy and artificial ventilation. Anesthesia was deepened with pentobarbital (12 mg/kg body weight per h). The carotid artery was cannulated for continuous pressure monitoring. The arteria saphena magna, which corresponds to the anterior tibial artery in humans and is the main arterial supply to the lower limb and foot in the rabbit, was exposed just above the ankle and cannulated with sterile polyethylene heparinized tubing. These tubings were connected to a Statham P23DC pressure transducer (Statham, Spectramed) for measurement of peripheral pressures (PP). After heparinization with 5000 U heparin, the left femoral artery was exposed and cannulated with sterile polyethylene catheter for the microsphere reference sample. After cannulation of the abdominal aorta a pump-driven shunt between the arteria carotis and the distal aorta was installed to perfuse both hindlimbs. A flow probe was installed to measure total flow to both hindlimbs.

2.3. In vivo pressure–flow relations

Maximum vasodilation was achieved by injecting adenosine (1 mg/ml; Sigma Chemical Company, St. Louis, MO). After stabilization of peripheral and central pressures both legs were perfused at six different pressures. The different perfusion pressure levels were generated with a roller pump (Stoeckert) installed in the shunt between the carotid artery and abdominal aorta. Peripheral pressures and collateral flows were measured under maximal vasodilation (adenosine) using Statham pressure transducers. For each pressure level, differently labeled fluorescent microspheres (scarlet, crimson, red, orange, green or blue-green; diameter, 15 μm; Molecular Probes, Eugene, OR, USA) were injected into the mixing chamber, which was installed in the carotid-abdominal aortic shunt. All recordings were stored on a computerized recordings system (MacLab, Macintosh) from which they were retrieved for further processing.

2.4. Counting of microspheres

The following muscles were dissected from the leg, quadriceps, adductor longus, adductor magnus, gastrocnemius, soleus, and peroneal muscles. Each muscle was divided into three consecutive samples from the proximal to the distal end. The whole muscle before and after sectioning was weighed and cut into 1–2 g pieces. The muscle samples were then placed loosely into 12 × 75 mm polystyrene tubes (Becton Dickinson & Co, Lincoln Park, NJ) and 3 ml of sodium dodecyl sulfate (SDS) solution [SDS solution, 1% SDS (Boehringer Mannheim Corp.); 0.5% sodium azide (Sigma Chemical Company); and 0.8% tween-80 (Fisher Scientific, Fairlawn, NJ) in 50 mM pH 8 tris buffer (Sigma Chemical Company)], proteinase K solution (2mg/ml; Boehringer Mannheim Corp.) and of blue microspheres as internal standard was added (diameter, 15μm, Molecular Probes). Each tube was capped and secured in a shaking water bath for 24–48 h. The samples were then centrifuged at 1000 × *g* for 45 min, the supernatant was pipetted off and the pellet was resuspended in 1 ml phosphate buffered saline (PBS; pH 7.4). The probes were rigorously shaken before FACS analysis. The microspheres were counted using a flow cytometer (FACS-Calibur) equipped with a second laser and a detector for a fourth flourescence. Flows for each sample were calculated from the number of microspheres in the sample, the respective microspheres count in the reference sample, the internal standard in the sample, internal standard in the reference sample, the weight of the reference sample and the time during which the reference sample was withdrawn using an equation as previously described.

2.5. Calculation of conductances

In our model collateral arteries, which develop after femoral artery occlusion in typical corkscrew formation, supply blood to the distal adductor region and the lower leg. We measured systemic pressure (SP) and PP. Venous pressure was equal to atmospheric pressure (AP). Since arterial resistances are much lower than collateral and peripheral resistances, they can be neglected. SP represents the pressure at the stem region of the collateral arteries. PP is the pressure at the reentry region and is identical to the pressure head of the circulation in the lower leg; AP is the pressure at the venous end of the peripheral circulation. Collateral flow is equal to the sum of flows to the tissue of the distal adductor plus the flow to the tissue of the lower leg. Collateral resistance was defined as pressure difference between SP and PP divided by the flow going to the distal adductor in the lower leg. The reciprocal values of these resistances represent the collateral conductance.

Because a positive pressure intercept is observed even at maximal vasodilation, all conductances were calculated from the slope of pressure–flow relations.

2.6. Postmortem angiography

After maximal vasodilatation with adenosine, legs were perfused with Krebs–Henseleit buffered saline in a warmed waterbath of 37 °C for 1 min at a pressure of 80 mmHg, followed by perfusion for 8–10 min at 80 mmHg with contrast medium based on bismuth and gelatin according to a formula developed by Fulton (as previously described) [35]. Subsequently, the contrast medium was allowed to gel by placing the limbs on crushed ice for 60 min. Angiograms were taken at two different angles in a Balteau radiography apparatus (Machlett Laboratories) using a single-enveloped Structurix D7DW film (AGFA). The resulting stereoscopic pictures allowed analysis of collateral growth in 3-D.

2.7. Quantification of collateral arteries

To differentiate between normal arteries and collateral vessels for further quantification, we used Longland's definition of collateral arteries. Stem, midzone and reentry were identified under stereoscopic viewing using a 3-fold magnification of our angiograms. Collateral arteries consisted of vessels whose stem either branched from the arteria circumflexa femoris lateralis or from the arteria profunda femoris. The midzone was of almost uniform length. Reentry of the collaterals usually descended into the arteria genus descendens or into the arteria caudalis femoris. Only about 10% of the collateral arteries originate from other vessels, e.g. from the a. iliaca externa or from the a. iliaca interna. Collateral vessels were marked after counting to make sure that no vessel was counted twice. We used a total magnification of 3^2 to measure the diameter of the vessels to an accuracy of 0.1 mm.

2.8. Detection of apoptotic monocytes

To evaluate the apoptosis rate of circulating monocytes we infused either albumin ($n = 3$), MCP-1 ($n = 3$) or GM-CSF ($n = 3$) or the combination of both ($n = 3$) via osmotic minipumps into the collateral circulation of the rabbit hindlimb. During this time period (7 days) blood samples from each animal were obtained. Rabbit peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll–Hypaque (Pharmacia & Upjohn, Freiburg, Germany). Monocytes were determined by FACS analysis via CD14 antigen expression and binding of FITC-conjugated Annexin-V (Alexis Corporation, New York, NY) was used for detection of apoptotic cells. An anti-annexin-V-antibody (Alexis Corporation) conjugated with FITC was used for detection of apoptotic cells. The anticoagulant annexin V is a member of a family of structurally related proteins that exhibit Ca^{2+}-dependent phospholipid binding properties. Annexin V binds to various phospholipid species with highest affinity for phosphatidylserine (PS). In normal cells, PS is situated on the inner leaflet of the plasma membrane. When programmed cell death occurs, PS is translocated to the outer layer of the membrane, i.e. the cell surface. This occurs in the early phases of apoptosis during which the cell membrane itself remains intact. Furthermore apoptosis was confirmed by Hoechst-33342 staining and light microscopy (see below).

2.9. Quantitative analysis of monocyte apoptosis by fluorescence microscopy

Fluorescent DNA-binding dyes were used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells to be analyzed were stained with Hoechst 33342 (5 μg/ml), added to the culture medium for 20 min, at 37 °C. The media and the PBS rinses were collected and the cells were trypsinized. Media, PBS, and trypsinized cells were pooled and collected by centrifugation at 1200 rpm for 5 min at 4 °C. Cell pellets were resuspended in a small volume (50 μl) of serum-containing medium with 1 μg/ml Hoechst 33342 and 5 μg/ml propidiumiodid (PI). An aliquot (25 μl) was placed on a glass slide, covered with a glass coverslip, and viewed under fluorescence microscopy. Individual nuclei were visualized at 400 × to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells.

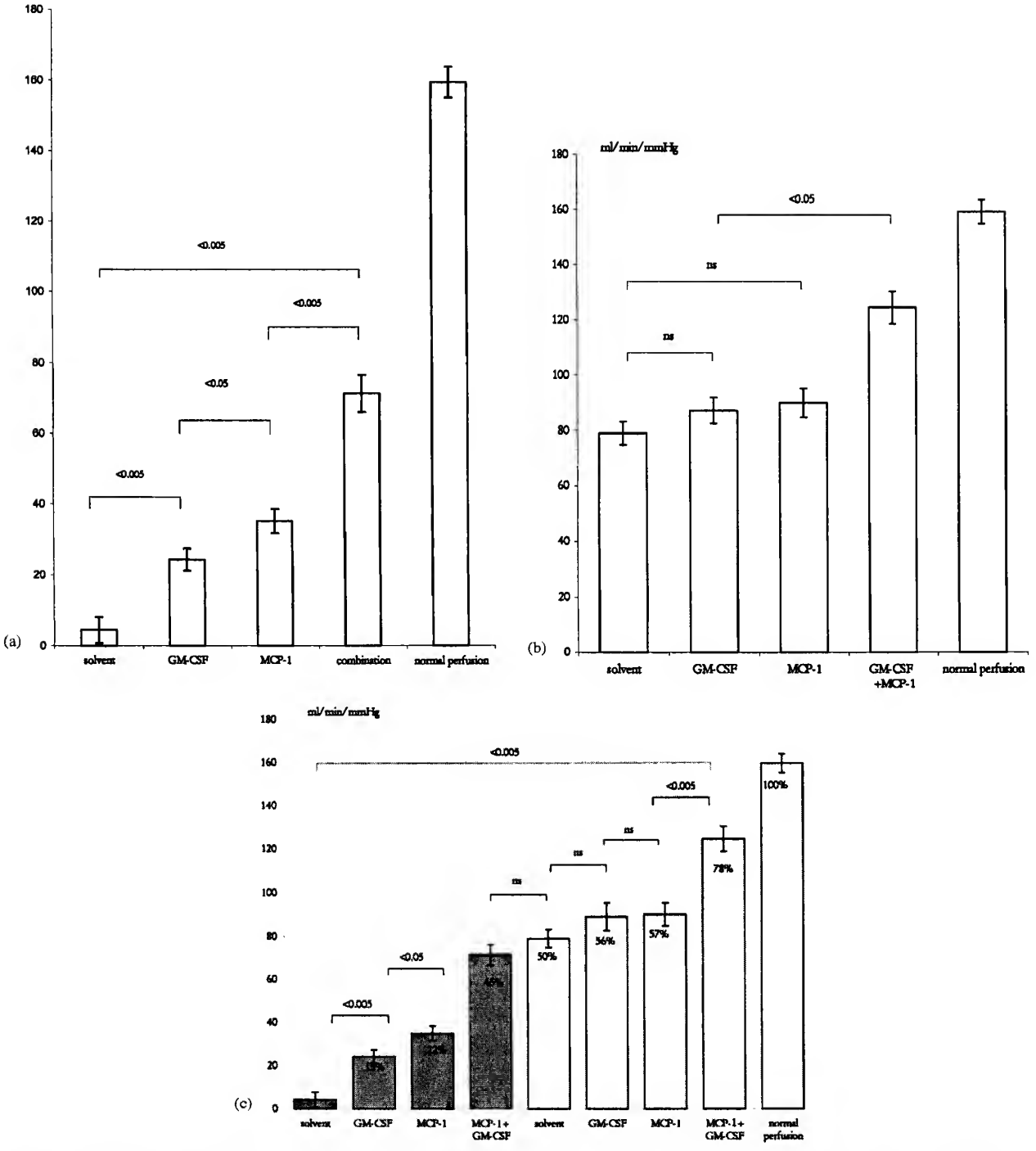

Fig. 1. (a) Collateral conductance first week after femoral artery occlusion with continuous infusion with either MCP-1, GM-CSF, combination of both or solvent over 7 days; (b) collateral conductance after 3 weeks of femoral ligation and 1 week treatment (fourth week) with either MCP-1, GM-CSF, combination of both or solvent (conductance units in ml/min per mmHg); (c) collateral conductance after treatment with MCP-1, GM-CSF, combination of MCP-1 plus GM-CSF as compared with normal perfusion. ▩, Ligation of femoral artery for 1 week, during this time period continuous infusion of solvent, GM-CSF, MCP-1 or the combination of MCP-1 plus GM-CSF. □, Ligation of femoral artery for 3 weeks, in the fourth week continuous infusion of solvent, GM-CSF, MCP-1 or the combination of MCP-1 plus GM-CSF.

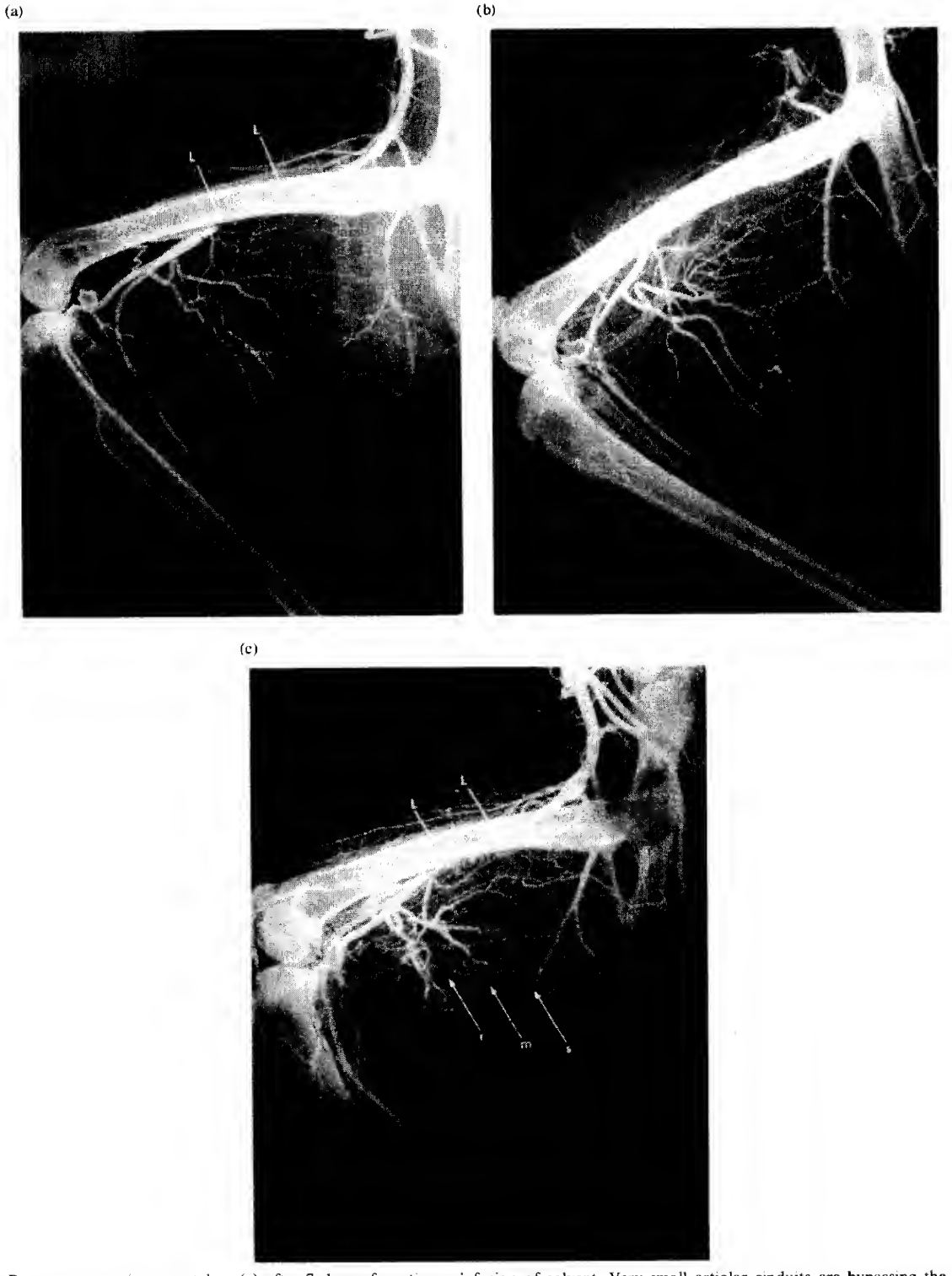

Fig. 2. Postmortem angiograms taken (a) after 7 days of continous infusion of solvent. Very small artiolar cinduits are bypassing the site of occlusion (L = ligation of femoral artery). (b) After 7 days of continous infusion of granulocyte-macrophage colony-stimulating-factor (GM-CSF), the number as well as the density of the collateral arteries has increased significantly. (c) After 7 days of continous infusion of monocyte chemoattractant protien-1 (MCP-1), the stem (s), midzone (m) and reentry (r) can be clearly identified.

Table 1

Peripheral blood:	Solvent-group	GM-CSF-group	
Monocytes	4.1 ± 0.8%	3.8 ± 1.0%	ns
Lymphocytes	35 ± 12%	36 ± 9%	ns

2.10. Transmigration assay

In order to rule out that GM-CSF promotes arteriogenesis via chemoattraction of monocytes, we tested adhesion to human umibilical venous endothelial cells (HUVECs). HUVECs were cultured in endothelial cell medium (Promocell, Heidelberg, Germany) to confluence on Millicell polycarbonate membranes (poresize, 3 μm; Millipore, Eschborn, Germany). Confluence was observed by HE-staining. For performing transmigration MCP-1 and/or GM-CSF was diluted in macrophage serumfree medium and placed into the lower compartment. Isolated monocytes were diluted in macrophage serum-free medium to a concentration of 10^6 cells per ml and placed into the upper compartment. After an incubation period of 3 h the membranes including the upper compartment were removed and transmigrated cells were further cultivated for 12 h. To analyze transmigration performance migrated cells were counted in a CASY 1 (Schaerfe Systems, Reutlingen, Germany) and apoptosis was quantified by FACS analysis using FITC-conjugated Annexin V (Alexis, Switzerland).

2.11. Adhesion assay

HUVECs were cultured to confluence on microtiterplates and stimulated for 4 h with TNF-α (10 ng/ml; Sigma, Deisenhofen, Germany). The culture medium was removed and stimulated monocytes were incubated in macrophage serum-free medium (10^6 cells per well) for 1 h. After this period the supernatant was removed and the microtiterplate was washed three times with PBS (pH 7.2). For analysis adherent monocytes were counted. Results are given as adherent monocytes per well.

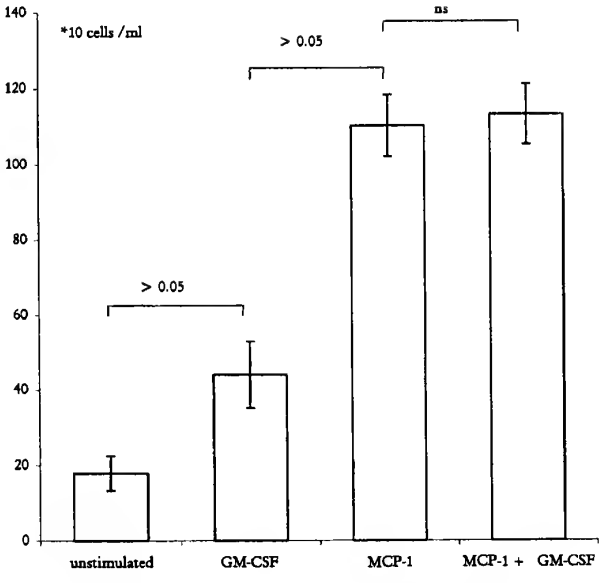

Fig. 3. Number of monocytes adhering to HUVECs.

2.12. Evaluation of apoptosis of adhering monocytes

2.12.1. FACS-analysis

Human isolated monocytes were allowed to attach to HUVECs (see above). Afterwards microtiterplates were carefully washed with PBS to remove non-adherent monocytes. Twelve hours later adherent monocytes were moblized from the endothelial layer (15 min at 4 °C) and immediately transferred to FACS-Analysis

Table 2
Visible collateral arteries

	solvent	GM-CSF	MCP-1	GM-CSF+ MCP-1
number of collateral arteries	15.4 ± 2.2	25.6 ± 3.6	30.1 ± 3.3	34.6 ± 2.1
	*	*	*	

*P-value less than 0.05.

(CD 14 positive cells were gated and quantified for apoptosis staining (Annexin-V FITC).

2.12.2. Histological analysis

Monocytes attached to HUVECs were stained with antibodies against CD68 (red signal), endothelial cells were stained with antibodies against CD 34 (green signal). Apoptotic cells were stained according to the Tunel-protocol (yellow signal).

2.12.3. Statistical analysis

Data are described as mean ± S.D. Differences among data were assessed using unpaired Student's *t*-test for intergroup comparisons and Mann–Whitney rank-sum test for unequal variances. Values of $P \leq 0.05$ were required for assumption of statistical significance.

3. Results

In agreement with observations by Schaub et al. [29] our testing of blood samples (FACS) and blood smears (cytology) confirmed that NZWR are 'non responders'. There was no significant increase in the number of circulating granulocytes and monocytes due to the GM-CSF treatment (Table 1).

3.1. Radiographic findings

Angiograms taken from hindlimbs of animals treated with GM-CSF from group 1 (Fig. 2b) as well as MCP-1 (group 1) (Fig. 2c) showed a remarkable increase in the number (Table 2), diameter and density of these collateral vessels as compared with animals with vehicle treatment (Fig. 2a). The typical morphology of collateral arteries can be clearly identified: (stem (s), midzone (m), reentry (r), L, site of femoral artery ligation).

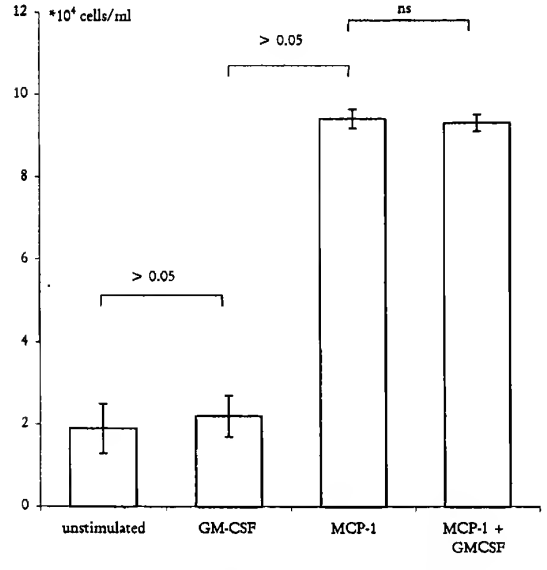

Fig. 4. Number of transmigrated monocytes.

3.2. In vivo pressure–flow relations

3.2.1. Group 1: early treatment (see above)

Collateral conductance was significantly higher after 7 days of occlusion in animals treated with GM-CSF compared with animals without this treatment (Fig. 1). GM-CSF plus MCP-1 treatment showed an increase in collateral conductance of more than 40% (compared with maximal perfusion without ligation of the femoral artery). These values could not be reached by maximal MCP-1 application alone (≥ 8–10 μg/day). The highest conductance levels after single-treatment with MCP-1 was 33 conductance units.

3.2.2. Group 2: late treatment (see above)

7 day treatment with GM-CSF or MCP-1 in the **fourth week after ligation** is not sufficient to significantly improve arteriogenesis. In contrast the concomitant application of MCP-1 plus GM-CSF enhances arteriogenesis to values of about 70% of normal maximal perfusion values.

3.2.3. Evaluation of monocyte adhesion

Average adherence of untreated human monocytes to HUVECs was low (20 cells per well) and comparable to GM-CSF primed monocytes (44 cells per well). In contrast, monocyte adhesion after incubation with MCP-1 increased 6-fold to 110 adhered cells per well (Fig. 3), which could not further be enhanced by concomitant GM-CSF application. While the number of adhering monocytes in the GM-CSF plus MCP-1 group was comparable to the single MCP-1 treatment, the percentage of protection from apoptosis was significantly higher when MCP-1 treated monocytes were also being pretreated with GM-CSF.

3.2.4. Evaluation of monocyte transmigration

The use of MCP-1 led to a significant increase in monocyte transmigration (9.2×10^4 cells per ml) whereas GM-CSF* did not induce monocyte transmigration (comparable to untreated monocytes° (*1.7×10^4 vs. °1.6×10^4 cells per ml) (Fig. 4). Concomitant application of GM-CSF with MCP-1 did also not increase the number of transmigrated monocytes.

3.2.5. Detection of monocyte apoptosis via light microscopy and FACS analysis

In the GM-CSF treated monocyte group the rate of apoptosis was significantly reduced. Annexin V-dependent fluorescence decreased from a mean value of 34.68 in the control group to 14.08 in the GM-CSF-treated

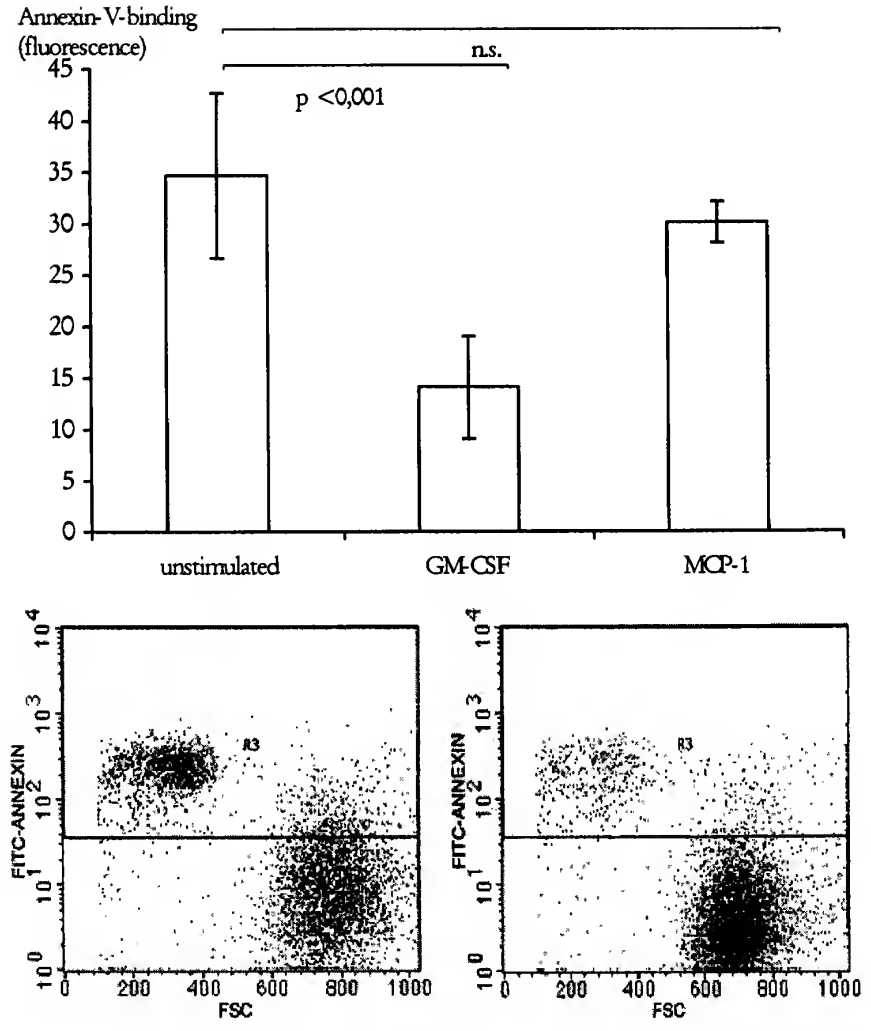

Fig. 5. Upper part, detection of monocyte apoptosis via FACS analysis (Annexin V binding); lower part, dot plotting of apoptotic monocytes (FACS).

group. Additionally, the GM-CSF-treated group also showed a significantly lower apoptosis rate when compared with a MCP-1 treated group (mean fluorescence, 24.5) (Fig. 5). This was confirmed via qualitative fluorescence microscopy of Hoechst-33342-stained cells (Figs. 6 and 7). Apoptotic monocytes showed the characteristically modified nuclei (Fig. 8). FACS-isolated Annexin V positive monocytes showed typical patterns of apoptosis (Fig. 9) as compared with non-apoptotic monocytes (Fig. 10). In the macrophage group a similar reduction of apoptosis after GM-CSF treatment was observed via FACS-analysis. Fig. 13 shows the typical morphology of a non-apoptotic macrophage (electron microscopy sections kindly provided by Dr Keisuke Suzuki). The nuclear pattern of this macrophage is not condensed, the cell exhibits large amounts of endoplasmic reticulum, indicating high protein turnover. Fig. 14 (electron microscopy section) shows an apoptotic macrophage with margination and condensation of chromatin and fragmented nuclei. Morphological signs of cell activity are only weakly visible (endoplasmic reticulum) while the cell membrane is intact.

3.2.6. Histological analysis of monocyte apoptosis during adhesion

Figures 11 and 12 show monocytes attaching to confluent HUVECs. After attachment microtiterplates were carefully washed with PBS. Adherent monocytes can be identified with antibodies against CD68 (red). Apoptotic monocytes exhibit a condensed pattern of chromatin. These monocytes were Tunel positive (m°). Endothelial cells can be identified in the background.

4. Discussion

Circulating monocytes originate from pluripotent hemopoietic progenitors in the bone marrow and provide a broad spectrum of physiological and pathophysiological properties. Once they leave the bloodstream, fractions of monocytes serve as precursors for several other cells with phagocytic function (Kupffer cells of liver, osteoclasts of bone, etc.) or antigen presenting dendritic cells. Others mature into tissue macrophages and are responsible for removal of debris as well as defense against invaders such as fungi and bacteria, which cannot be dealt with effectively by neutrophils (unlike neutrophils, macrophages are able to regenerate their lysosomal granules and may thus have a longer lifespan than neutrophils) [37]. However, flow cytometry and functional monocyte assays have shown that monocytes are a very heterogenous group of cells. Only 30–40% respond to chemoattractants, the expression of α and β-integrins and of the cell-surface HLA-DR antigen varies significantly[11]. The capacity to produce reactive oxygen species is very different suggesting that only subpopulations of monocytes are able to participate in specific immune responses [38]. Moreover, it has recently been reported that a subset of

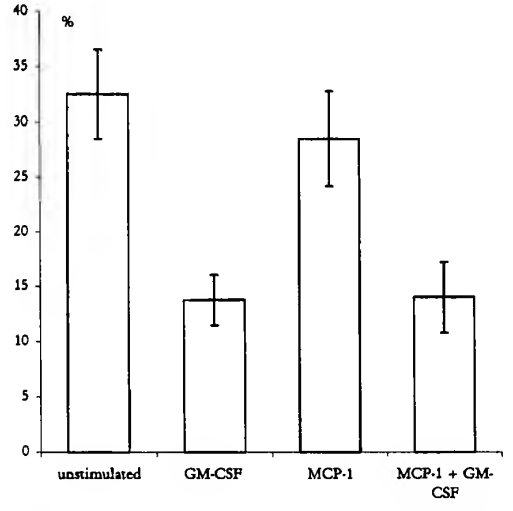

Fig. 6. Verification of monocyte apoptosis via Hoechst-staining (percentage of apoptotic cells via light microscopy).

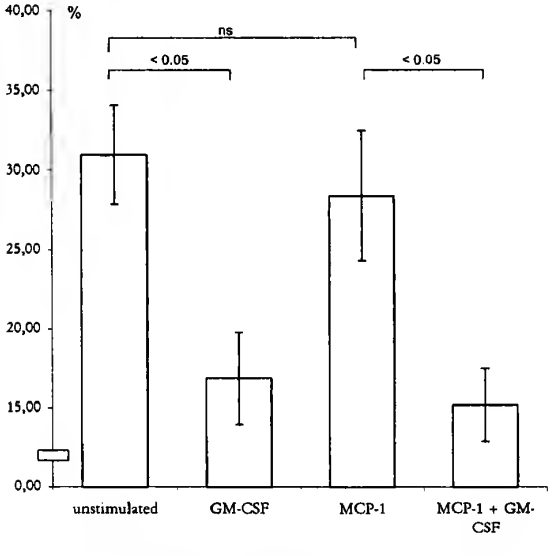

Fig. 7. Percentage of apoptosis of adhering monocytes.

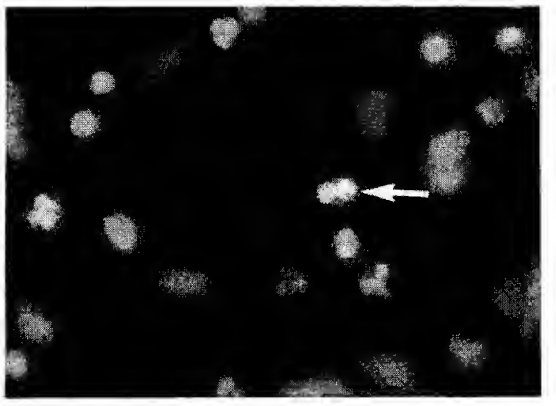

Fig. 8. Nuclear chromatin morphology of monocytes was analyzed via a quantative index of apoptosis (Hoechst 33342 and propidiumiodid staining). Individual nuclei were visualized at 400x to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells (arrow).

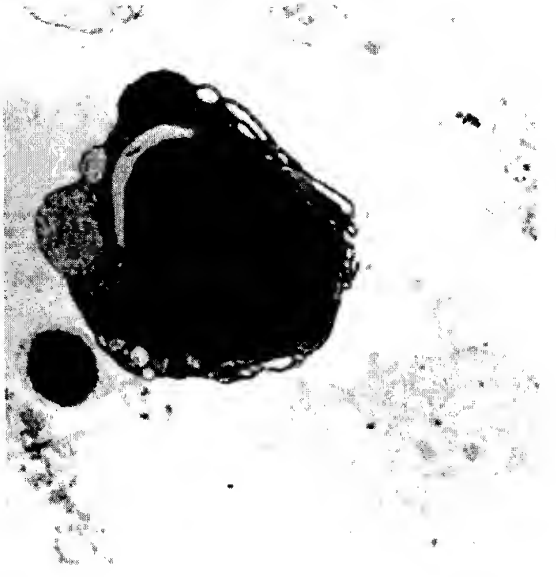

Fig. 9. Apoptotic monocyte with margination and chondensation of chromatin, fragmented nuclei and intact cell membrane.

CD34-monocytes contains a fraction with the potential to differentiate into an endothelial phenotype [39]. Despite this heterogeneity all monocytes derive from common hematopoietic precursor cells and require a complex array of biologically active proteins (in particular CSFs), which influence their development and survival.

The main findings of our study are that chronic intra-arterial infusion of GM-CSF stimulates the development of arterial collateral blood vessels (arteriogenesis) following femoral artery occlusion. These are more numerous on angiograms and their ability to conduct blood had increased by a factor of 5-fold. The mechanism of action is the prolonged survival of monocytes-macrophages, known to play a decisive role in arteriogenesis [1–8]. Furthermore GM-CSF is a powerful adjunct to the treatment with CC-chemokines (MCP-1) to induce arteriogenesis. The highest value of collateral conductance after 1-week high-dose MCP-1 alone treatment reached 20% of normal perfusion values whereas the combination therapy of MCP-1 plus GM-CSF was twice effective (conductance more than 42% of normal perfusion values). For the exogenous supply of several angiogenic growth factors only a brief time window is available for action [3], usually within hours or days following arterial occlusion. This limits their therapeutic power in subjects with a stable but functionally deficient collateral circulation. Therefore, we tested whether growth of collateral arteries can be re-started after long-term femoral ligation in the rabbit. Three weeks after ligation of the femoral artery both MCP-1 as well as GM-CSF were infused for 1 week into the matured collateral circulation. Both single treatments did not improve perfusion markers as compared with the natural course of collateral artery growth. However, when GM-CSF and MCP-1 were infused simultaneously the effects on arteriogenesis were additive on angiograms as well as on conductance. The effect of the combined treatment at 4 weeks after femoral occlusion was about 80% of normal maximal conductance of the artery that was replaced by collaterals, a result that was not reached before by any other experimental treatment.

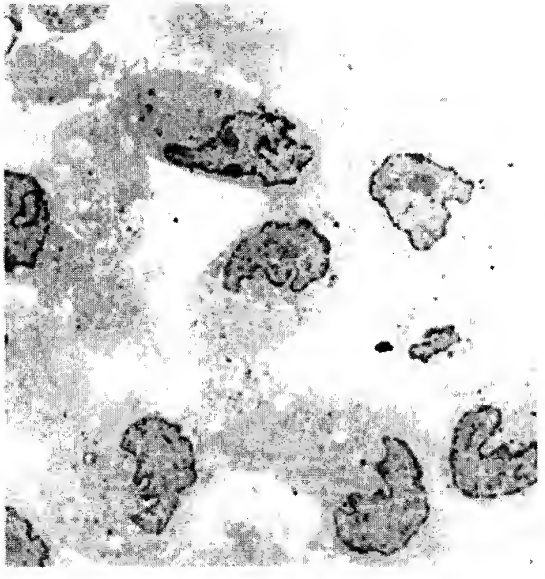

Fig. 10. Nuclear pattern of non-apoptotic monocytes viewed under light microscopy. The cells show no condensed chromatin.

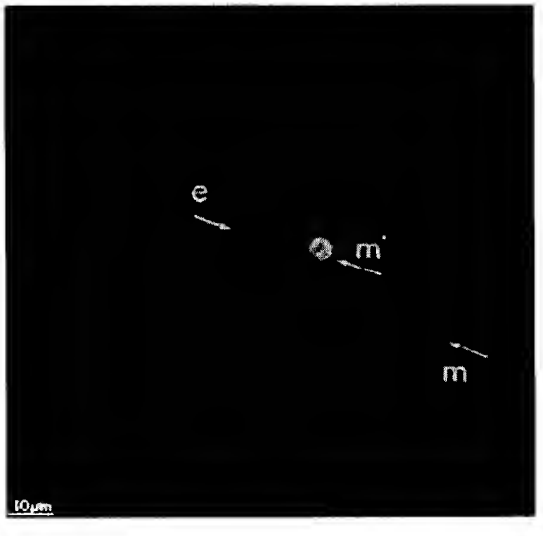

Fig. 11. In-vitro adhesion assay: double staining for adhering macrophages with CD 68 (m), endothelial cells (e) and apoptosis after the tunel-protocol (m°).

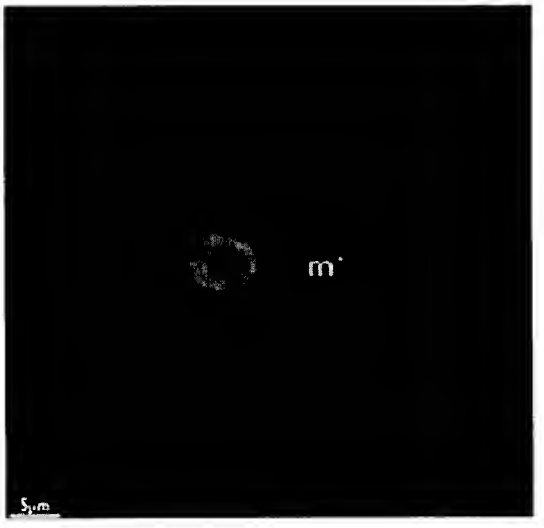

Fig. 12. In-vitro adhesion assay: magnification of an apoptotic monocyte.

In previous studies we had shown that stimulation of monocyte function by LPS [1] and by infusion of the monocyte chemoattractant factor MCP-1 [5,6] greatly increased arteriogenesis. Arteriogenesis differs from angiogenesis in several aspects, the most important being the dependence of angiogenesis on hypoxia and the dependence of arteriogenesis on inflammation. Collateral arteries grow surrounded by tissue that is not

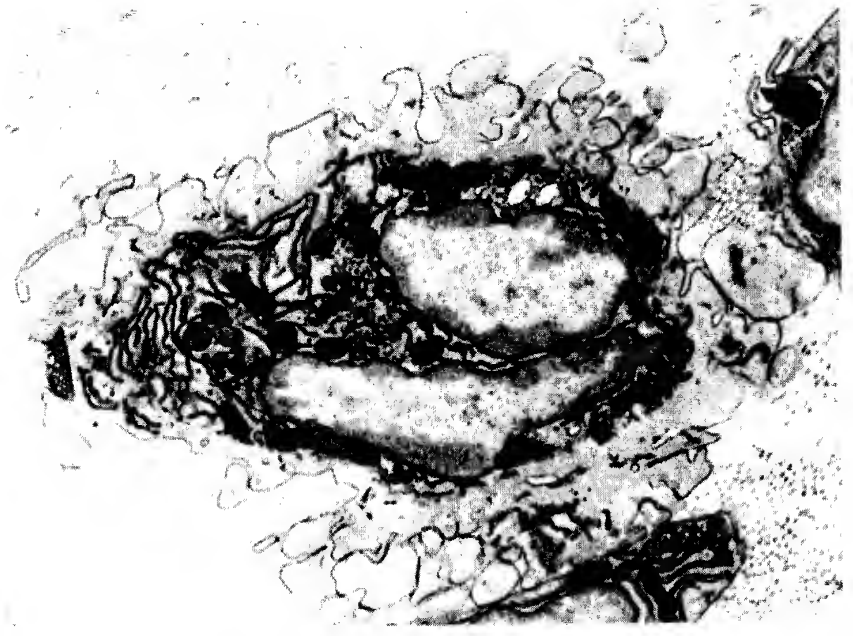

Fig. 13. Nuclear pattern of non-apoptotic macrophage. This macrophage possesses many endoplasmic reticulum, indicating its activity in protiens synthesis (electron microscopy: × 5000, bar = mm).

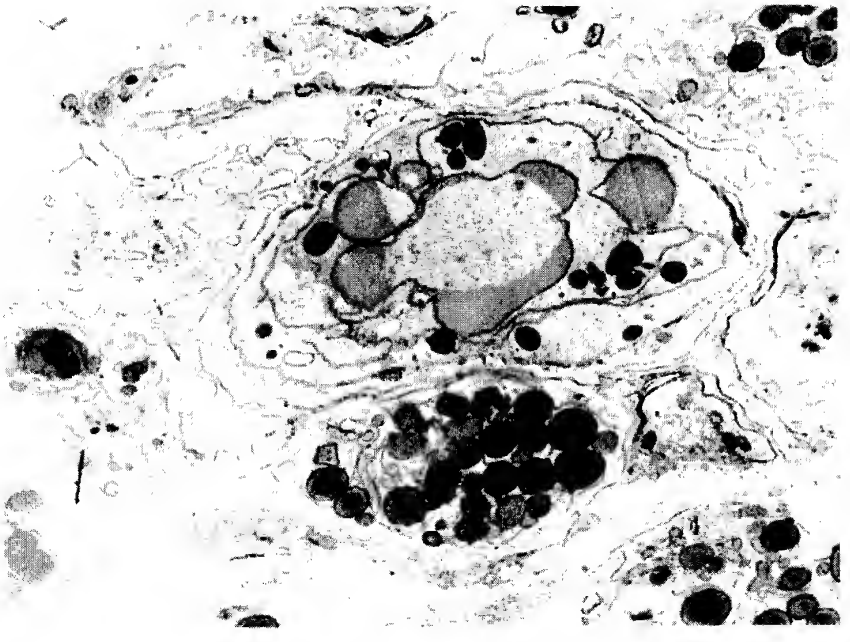

Fig. 14. Apoptotic macrophage with margination and chondensation of chromatin, fragmented nuclei and intact cell membrane (electron microscopy: × 5000, bar = mm). The endoplasmic reticulum is only weakly visible.

ischemic: its resting blood flow is not decreased, its ATP and PCr content is normal and hypoxia-induced gene transcription (LDH-A, VEGF) is not activated [34]. Arteriogenesis is by far the most efficient adaptive mechanism for the survival of ischemic limbs or internal organs like the heart because of its ability to conduct, after adaptive growth, relatively large blood volumes per unit of time. An increased number of capillaries, the result of stimulated angiogenesis, is unable to do that [2,3,8]. The inflammatory environment, which is necessary for arteriogenesis is created by homing of circulating cells to shear stress activated endothelium, which express MCP-1. These circulating cells are mainly monocytes (derived from hemopoietic stem cells) and lymphocytes but also basophiles that transform in the tissue into mast cells where they produce vasoactive substances [2,36].

Our present study supports the inflammatory paradigm of arteriogenesis because GM-CSF prolongs the duty cycle of monocytes-macrophages and protects high numbers of MCP-1 attracted monocytes from apoptosis and thereby enhances their arteriogenic activity. CSFs are essential for the survival of macrophages and significantly influence the rate of apoptosis of circulating and adherent blood monocytes as well as resident tissue macrophages. Monocytes circulate in the blood for 12–72 h. Those cells that are attracted to the site of inflammation by chemotactic factors will, nonetheless, die by apoptosis unless provided with specific survival signals [18]. It is important to recognize that programmed cell death occurs without inflammation and is a normal physiological response by many eukaryotic cells to factors and conditions that are, as yet, poorly defined [13]. In the normal resolution of an acute inflammatory response apoptosis of monocytes and neutrophils is essential to maintain immune homeostasis and limit inappropriate host tissue damage by decreasing white blood cell tissue load, function, and release of phlogistic reactive oxygen species and proteases. Monocytes are known to undergo spontaneous apoptosis upon leaving the circulation unless provided with specific survival signals. Interestingly, factors that are needed for the recruitment of monocytes into inflammatory lesion, namely TGF-1, bacterial peptides and MCP-1 have little or no effect on monocyte survival [18]. Taken together these data are consistent with our observations. The increase of shear stress initiates the recruitment of monocytes by upregulation of adhesion molecule expression on the endothelial lumen of the collateral vessel. Simultaneously the production of GM-CSF by the endothelium is rapidly increased [33]. Since the chemoattractive action of GM-CSF on monocytes is rather weak the monocyte recruitment is mediated by a direct effect of GM-CSF on monocyte activation, proliferation, differentiation and motility, and secondarily, by chemoattractant molecules released in response to the locally administered GM-CSF. The present in vivo study also indicates that GM-CSF is able to significantly prolong the life-span of monocytes in vivo via a reduction of apoptosis. These data were confirmed by our in vitro studies, showing the protective effect of GM-CSF on monocytes and macrophages. The demonstration of a potent therapeutic effect of exogenously administered GM-CSF on collateral artery growth in this model confirms our previous reports about the central role of monocytes during the rapid growth of muscular collateral arteries (arteriogenesis). Since CSFs have remarkable antiatherogenic effects [24,26–31], GM-CSF might provide an important mechanism, other than chemoattraction [18], to significantly enhance arteriogenesis in atherosclerotic subjects. It is worth noting that macrophages can in principle be their own source of regulatory cytokines such as GM-CSF, which in turn upregulate their own production of cytokines [18]. Since this mechanism is clearly suboptimal, substitution therapy, as in our study, is more effective. Finally exogenous GM-CSF may also release hemopoietic stem cells from the bone marrow that may be even more potent stimulators of arteriogenesis than circulating monocytes as our preliminary studies imply (in preparation).

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"Tissue Need" and Limb Collateral Arterial Growth

Skeletal Contractile Power and Perfusion During Collateral Development in the Rat

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Among the factors that might influence collateral arterial growth after arterial occlusion, the capacity to deliver blood flow in relation to metabolic need and work performance are obvious candidates. In this study in rats after superficial femoral artery ligation, we assessed collateral arterial growth (by arteriography), basal and peak limb blood flow during acetylcholine-induced vasodilation (by electronic drop counting), pressure–flow relations, and contractile power of the gastrocnemius muscle (force transduction during sciatic nerve stimulation) at intervals over 3 months after superficial femoral artery ligation. Basal and peak blood flow and muscle contractile power were clearly reduced 1 week after ligation but had returned to normal by 3 weeks. Major collateral arterial growth, however, progressed between 3 weeks and 3 months. The limb perfusion pressure–blood flow relation was still altered at 3 weeks, with blunting of the normal autoregulation, and became more normal by 3 months after superficial femoral artery ligation. Collateral arterial growth continues after blood flow adequate to maintain work performance has been restored and may reflect a response to more subtle abnormalities involving distal pressure delivery, evident in altered pressure–flow relations. (*Circulation Research* 1992;70:546–553)

KEY WORDS • **blood flow autoregulation** • **arteriography** • **skeletal muscle work** • **acetylcholine blood flow**

John Hunter, the first to recognize collateral arterial growth, suggested over 200 years ago that the force promoting collateral development is "tissue need."[1] The specific term he used, "the stimulus of necessity," was typically vitalist and thus required no further definition. This reasonable concept has often been repeated, need being translated into physiological and biochemical events related to tissue ischemia.[2–4]

Recent observations have also focused attention on the ischemic zone as a crucial source of information for arterial growth.[5–9] Hyperplasia, evident in increased tritiated thymidine incorporation into vascular elements, begins within 24 hours,[5–9] primarily in vessels near the ischemic zone and shows centripetal spread from there over the next several days.[6,7] This pattern has suggested that a complex communication system exists and that the ischemic zone is a crucial source of information for collateral arterial growth.[6,7] The hyperplastic process gradually grows quiescent,[5] but there is evidence of continued collateral arterial growth for many weeks.[3–5,9–11]

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What physiological forces might be responsible? Obvious candidates have included basal blood flow delivery, flow velocity, pressure delivery, blood flow in relation to support of metabolic work, and the products of metabolism.[4] Recent observations, however, have revealed a remarkably rapid return to normal of blood flow and the metabolic machinery to support work in rat skeletal muscle after superficial femoral artery ligation in the rat.[12,13] Because the time course was not congruent with observations on blood vessel growth, although none had been reported in the rat, we undertook to systematically assess blood vessel growth after superficial femoral artery ligation by arteriography in relation to basal limb blood flow, the maximal capacity of the limb blood supply to deliver flow during acetylcholine-induced vasodilation, and the capacity of the collateral-dependent skeletal muscle to contract. All are responsible surrogates for tissue need. All of these indexes of collateral arterial function had returned to normal within 3 weeks, but collateral arterial growth continued thereafter, suggesting that the forces at work to promote and regulate vascular growth lie beyond some of the intuitively obvious candidates.

Materials and Methods

The studies were performed in 192 Sprague-Dawley male rats weighing between 350 and 400 g. The very large number of rats studied reflected the fact that it was necessary to measure blood flow and contractile power and to obtain arteriograms in different groups of rats for technical reasons. Moreover, four time points

were assessed. During ligation of the superficial femoral artery to promote the growth of a collateral arterial tree, ether anesthesia was used; the artery to the left hind limb was ligated in a region just underlying the rectus abdominis muscle, about 1.5–2 cm above the superficial epigastric artery. The skin incision was closed with skin clips, and the animals were returned to cages for a follow-up study between 1 and 12 weeks after arterial ligation. In the normal controls (sham-operated group), a similar surgical procedure was used, but the artery was not ligated.

For follow-up studies, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the trachea was cannulated to maintain an airway. For blood flow studies, the right carotid and left femoral arteries were catheterized with PE90 siliconized polyethylene tubing (0.86 mm i.d., 1.27 mm o.d.; Clay Adams, Parsippany, N.J.). Catheter sizes were selected to provide the largest possible diameter and thus minimize resistance to flow. The jugular vein was also catheterized with a PE90 catheter for administration of fluids and supplemental doses of anesthesia as required. For arteriographic studies, the carotid artery catheter was advanced to the abdominal aorta and the femoral artery was not catheterized.

Blood flow to the hind limb was measured by means of a drop counting system that we have described in detail.[14] In brief, a disposable intravenous solution administration set (Travenol Laboratories, Inc., Deerfield, Ill.) was used, and the chamber was filled with liquid silicone of low viscosity (Dow Corning 200 Series; viscosity, 2.0 centistokes at 25°C). Heparin was administered intravenously (400 units). Arterial blood entered the system via the cannulated carotid artery and then traversed the chamber as a series of constant-sized drops, about three drops per milliliter. The outflow from the chamber entered the iliac artery at a level just above the origin of the collateral arteries to supply the vascular bed of the limb only via the collateral route. The active dead space of the system was 3.0 ml, which was filled at the beginning of the experiment with an artificial rat plasma. The artificial rat plasma was prepared from donor animals, which were bled. The red blood cells were separated, and the heparinized plasma was dialyzed for 8 hours against a Krebs solution to remove low molecular weight vasoactive factors. The protein and salts were stored dry after lyophilization and reconstituted with sterile distilled water just before the experiment.

A photocell (Grass Instrument Co., Quincy, Mass.) encompassed the drop chamber to register drop rate, representing blood flow to the limb. The system was calibrated with heparinized rat blood. Mean systemic arterial blood pressure and arterial perfusion pressure in the chamber were measured by means of Statham P23DC pressure transducers. Photoelectric and pressure transducer outputs were recorded continuously on a Grass polygraph. Drop rate was registered directly and also translated into a flow rate by means of an ordinate recorder, as previously described.[10]

After the surgical preparation was complete and the drop counting system was active, the rats, with rectal thermometers in place, were set on a heating pad to maintain a body temperature of 36–37°C. Perfusion pressure to the limb could be reduced by graded external compression on the inflow side of the drop counting system. The relation between perfusion pressure and blood flow was assessed by reducing perfusion pressure as a series of 10 mm Hg steps (see Figure 5) and assessing blood flow at each perfusion pressure between 1 and 3 minutes after the new pressure level had been reached, when blood flow achieved a new steady state. For purposes of presentation, complete autoregulation has been defined as a flow rate that is independent of perfusion pressure (slope not significantly different from 0) over a range of perfusion pressures of 30 mm Hg or greater.

Blood flow was assessed in 47 rats at rest and during acetylcholine-induced vasodilation obtained by injecting graded doses of 1.0–1,000 μg in 0.1 ml of saline as a series of boluses into a side arm of the catheter in the iliac artery. Acetylcholine induced the same peak blood flow as did maximal skeletal muscle work in both the normal and collateral-dependent limbs.[15] Perfusion pressure was measured via the same catheter. At higher acetylcholine doses a depressor response occurred, but the maximal blood flow increase occurred before blood pressure fell.

The ability of the skeletal muscles of the normal and collateral-dependent hind limbs to contract was examined in 37 animals. The sciatic nerve was exposed, and two electrodes were placed in contact with the nerve. A 0.5-cm-wide parafilm strip was placed loosely around the nerve, and both ends were sealed together to maintain separation of the electrodes. The wound was sutured, and the knee joint was fixed in position on a plexiglass board by means of a brass screw. The distal end of the muscle mass was attached to a Grass force transducer. Maximal force was generally obtained within 1–2 minutes and maintained throughout the balance of the 5 minutes of stimulation. The maximum force measured was the value used to describe work capacity of the limb. The muscle was adjusted to the optimal length for maximal isometric contraction. The muscles of the hind limb were stimulated supramaximally via the sciatic nerve with a Grass Model SD9c stimulator (1 msec, 3 V) at 2–3 Hz for 5 minutes. The muscles were allowed to recover for 15 minutes and restimulated to verify the result.

For arteriographic studies, meglumine diatrizoate or micropaque was infused into the abdominal aorta of 23 rats by pump injection of 0.8 ml over 15 seconds. Arteriograms were obtained with a Machlett microfocal spot tube with nominal size of 0.1 mm and a Giganto generator (Siemens Medical Systems Inc., Erlangen, FRG). Films were obtained with 3M Alpha-3 screens and with 3M XUD film. The conditions were 75 KVP, 80 MA, and 25 MS. The collateral arteries were too small for measurement by objective, computer-assisted methods. To ascertain whether there was continued growth of collateral arteries between the third week, when blood flow and work had returned to normal, and 3 months after superficial femoral artery ligation, we made a coded assessment of the arteriograms. One observer ranked, on a coded basis, each film set obtained at 3 weeks and 3 months and after acute occlusion.

Mean values have been presented with the standard error of the mean as the index of dispersion. Linear regression was used to assess the pressure–flow relation over selected pressure ranges. The coded assessment of arteriograms was analyzed by the Fisher Exact Test.

One-way analysis of variance (ANOVA) was used to assess the time course of flow changes after superficial femoral artery ligation. The null hypothesis was rejected when the probability was less than 0.05.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Results

One week after femoral artery ligation, basal limb blood flow was reduced from the normal 3.5±0.17 ml/min to 2.3±0.47 ml/min ($p<0.025$; Figure 1 [top panel]). Perfusion pressure was 85.1±3.2 and 86.0±7.3 mm Hg, respectively. By 3 weeks after superficial femoral artery ligation, basal limb blood flow had returned to 3.5±0.5 ml/min. Peak limb blood flow during acetylcholine infusion showed a similar pattern, falling from a normal 7.3±0.4 ml/min to 5.6±0.8 ml/min ($p<0.05$) at 1 week and returning to 8.8±2.0 ml/min at 3 weeks (Figure 1 [top panel]).

An essentially identical time course was seen in the contractile response of the gastrocnemius muscle to sciatic nerve stimulation after superficial femoral artery ligation (Figure 1 [bottom panel]). At 1 week, the contractile response had fallen from a normal 21.0±2.7 g to 12.9±2.0 g ($p<0.01$). By 3 weeks, the contractile response had returned to a normal value of 21.3±6.6 g, which exceeded the value at 1 week significantly ($p<0.025$).

The evaluation of the arteriograms for collateral vessel dimensions revealed a different time course. No collateral arteries were visible in the first 30–60 minutes after superficial femoral artery ligation (Figure 2). At 1 week, collateral arteries were visible but were very small and poorly defined (not shown). By 3 weeks, definite collateral arteries were routinely visible, but they remained small (Figure 3). In 10 rats studied at 3 months (Figure 4), all but two had better collateral arterial development than did the best of those studied at 3 weeks in a coded assessment, and none of the other nine studied at 3 weeks had a collateral arterial tree that was as well developed ($p<0.005$).

The anticipated blood pressure–flow relation was observed in the hind limb of normal rats (Figure 5 [top panel]). Autoregulation of blood flow was "complete" over a wide range of perfusion pressures, from 60 to 120 mm Hg. Blood flow showed no change with increases in perfusion pressure over that range. Conversely, below that pressure range, flow increased linearly from 0.4±0.2 ml/min at a perfusion pressure of 10 mm Hg to 4.3±0.3 ml/min at 60 mm Hg. Over the plateau pressure range, from 60 to 120 mm Hg, flow equaled 4.4±0.18 ml/min (slope=0, $r=0.015$, $F=0.010$).

On acute occlusion of the femoral artery, blood flow fell to minimal levels and remained there for 60 minutes.

In animals studied 3 weeks after occlusion of the femoral artery, there was a clear change in the pressure–flow relation (Figure 5 [middle panel]). There was no evidence of an autoregulatory break point. Blood flow apparently rose linearly with increasing perfusion pressure over the entire range of pressures studied, from 50 to 130 mm Hg (slope=0.0378±0.0069 (SD), $r=0.90$, $F=30.2$, $p<0.01$). Blood flow was identical to that in the normal animal at a perfusion pressure of about 100 mm Hg and rose to supranormal levels with higher pressures. Even over the more limited pressure range, from 70 to 130 mm Hg, a positive relation was identified

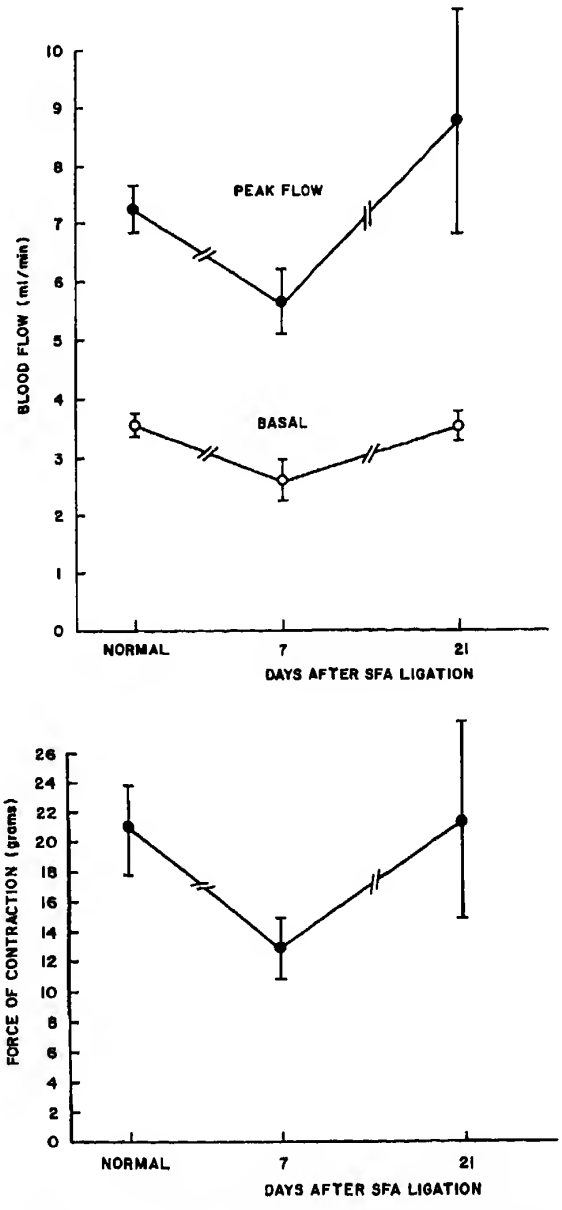

FIGURE 1. Top panel: *Basal limb blood flow and peak blood flow during acetylcholine-induced vasodilation in normal rats and rats studied 7 or 21 days after superficial femoral artery (SFA) ligation.* Bottom panel: *Peak force of contraction of the gastrocnemius muscle in response to sciatic nerve stimulation at the same time intervals. Note that all measures were reduced significantly at 7 days after SFA ligation but had returned to normal by 3 weeks.*

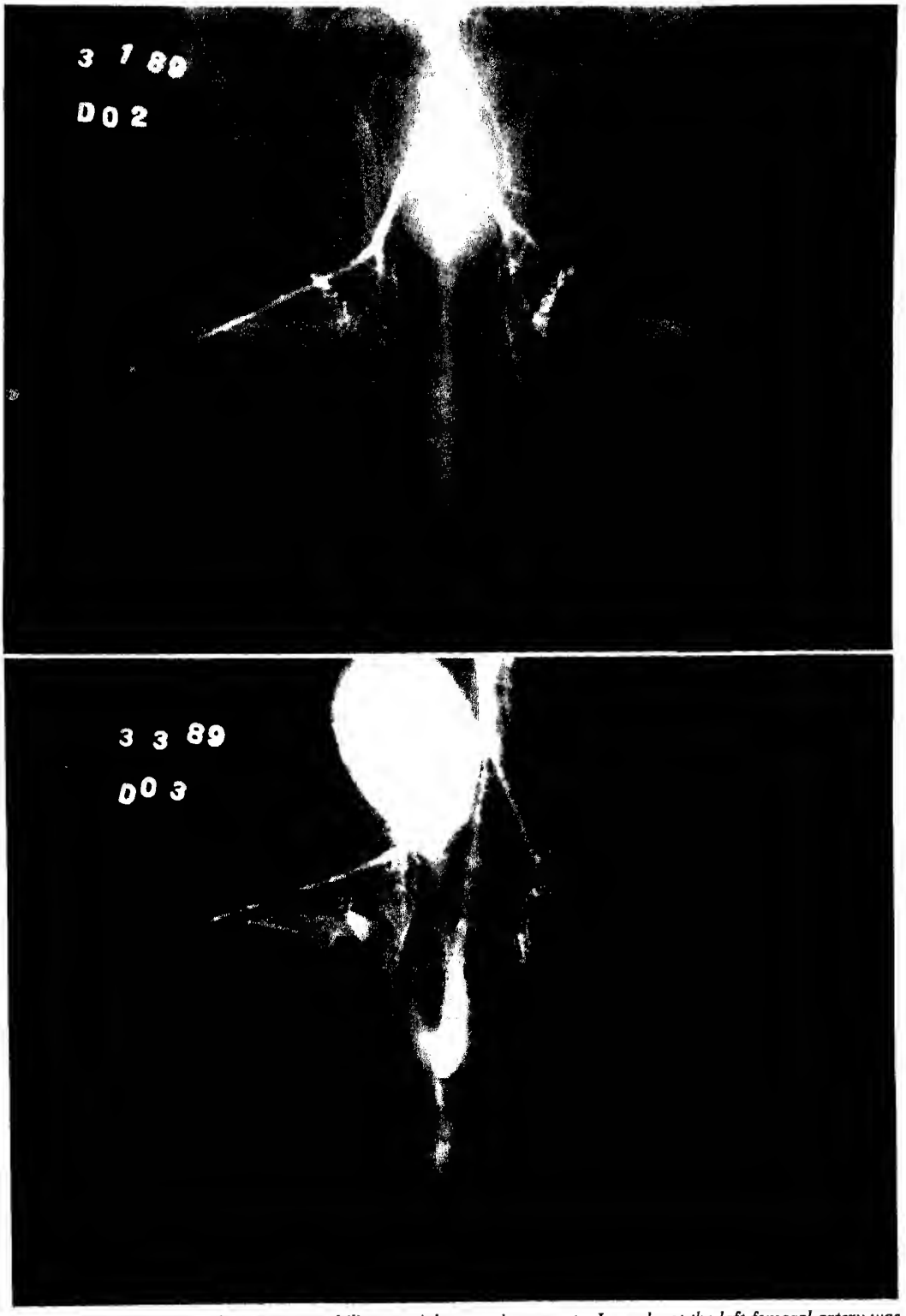

FIGURE 2. *Arteriograms of the lower aorta and iliac arterial system in two rats. In each rat the left femoral artery was ligated (arrow labeled a) 30 minutes before the arteriogram. Small amounts of contrast have reached the distal arterial tree, but distinct collateral arteries are not visible.*

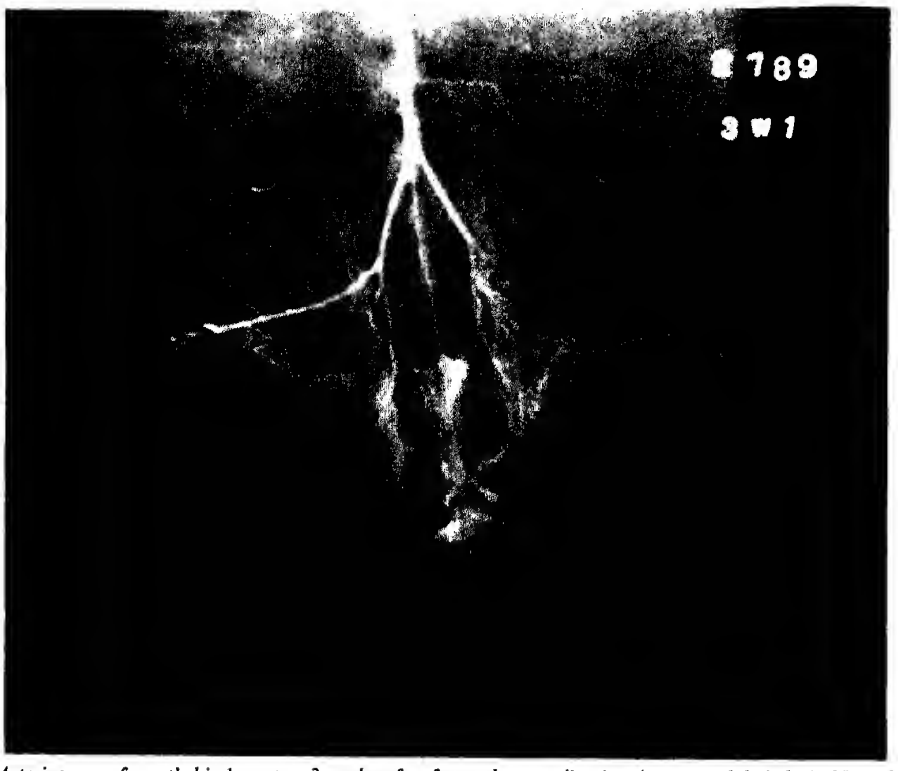

FIGURE 3. *Arteriogram of a rat's hindquarters 3 weeks after femoral artery ligation (at arrow labeled a). Note the presence of identifiable collateral arteries (small arrows) and the contrast-filled distal arterial tree (arrow labeled b). This was the most completely developed collateral arterial tree among 10 rats studied at this time.*

between pressure and blood flow (slope=0.023±0.007 (SD), r=0.82, F=10.2, p<0.001).

In animals studied 3 months after femoral artery occlusion, the results were somewhat ambiguous, but autoregulation of femoral blood flow was evident (Figure 5 [bottom panel]) over the pressure range of 60–110 mm Hg (slope=0, F=0.13). Blood flow over this pressure range averaged 4.03±0.3 ml/min (compare the top and bottom panels of Figure 5).

Discussion

The concept of tissue need (never fully defined) as the pivotal determinant of collateral arterial growth is long standing.[1] Our hypothesis in this study was that basal blood flow, maximal blood flow that a collateral tree can carry, and the capacity of the perfused tissue to perform work would provide an index for assessing whether a collateral arterial tree had satisfied tissue need. By all of these indexes, the collateral arteries were inadequate at 1 week. At that time they would not support muscle work, and basal and peak blood flow were reduced. Skeletal muscle contractile responses and blood flow, however, clearly had returned to normal by 3 weeks after superficial femoral artery ligation. These observations are in accord with earlier measurements of blood flow and muscle metabolism.[12,13] Collateral arterial growth assessed by angiography, on the other hand, showed a different temporal pattern. The collateral arteries, too small to be visualized by arteriography an hour after occlusion, clearly grew between the initial insult and assessment at 7 days but were still too small to be visualized clearly. By 3 weeks collateral arteries were routinely visible and well defined, but collateral vessels at 3 months were substantially larger than they had been at 3 weeks. The force responsible for continued collateral arterial growth, based on blood flow or capacity to perform mechanical work, is unlikely to be dependent on a signal emanating from residual tissue ischemia.

Of the physiological processes assessed in this study, only autoregulation of limb blood flow was still abnormal 3 weeks after superficial femoral artery ligation. Over the autoregulatory range of pressures in the normal animal, arteriolar dilation must compensate for a fall in perfusion pressure to maintain a constant blood flow. Complete autoregulation of blood flow in the normal limb and in normal skeletal muscle has been amply demonstrated in many species.[16–19] Although the precise factor responsible for shifts in arteriolar resistance with shifts in perfusion pressure has not been identified, most theses agree that the changes occur in vascular smooth muscle at the arteriolar level.[17] Complete autoregulation of blood flow over the blood pressure range of 60–120 mm Hg has been confirmed in this study. Indeed, the slope of the line relating flow to pressure was precisely zero. Three weeks after arterial ligation, when basal blood flow at normal perfusion pressure and peak blood flow in response to acetylcho-

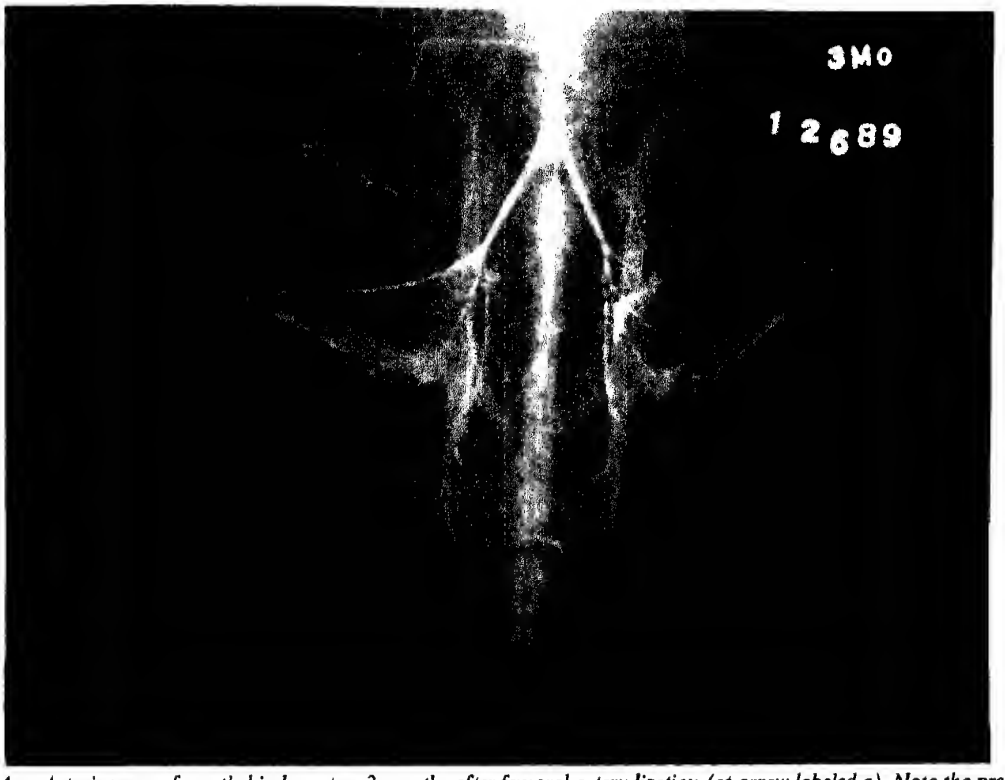

FIGURE 4. *Arteriogram of a rat's hindquarters 3 months after femoral artery ligation (at arrow labeled a). Note the presence of identifiable collateral arteries (small arrows) and the contrast-filled distal arterial tree (arrow labeled b). Note the better developed collateral arterial tree, which was evident in eight of 10 rats studied at 3 months in a coded assessment.*

line had returned to normal and when skeletal muscle metabolism was normal,[13] there was a clear alteration in the relation between blood pressure and blood flow. In this case there are two resistances in series, one provided by the collateral arteries and the other by the downstream arteriolar bed. In the presence of a significant resistance to blood flow provided by the collateral arteries, an alteration in the autoregulatory process would be no surprise. Indeed, one would anticipate that the reduced pressure delivery to the arteriolar level would have resulted in arteriolar dilation, thus limiting dilation as perfusion pressure fell further.[15] Although it is tempting to relate the continued growth of collateral arteries beyond 3 weeks to the disturbance in perfusion pressure–flow relations, there is no direct evidence in this study to support that mechanistic connection, and the relation must be considered speculative. The alternative possibility exists that the alteration in pressure–flow relation reflected vascular injury consequent to the ischemic process, with a time course of recovery that is slower than that of the skeletal muscle. Although possible, this seemed unlikely.

How can one account for a normal peak blood flow during acetylcholine infusion in this model? The only apparent explanation is that acetylcholine was effective in dilating the collateral arteries along with the distal arterioles. Because there has been substantial interest in enhanced responses of collateral arteries to serotonin, with the suggestion that the enhanced response reflects loss of endothelium-dependent relaxation,[20] the fact that acetylcholine was effective in dilating the collateral arteries would be of substantial interest. Clearly, this observation merits further investigation. Whatever the explanation, it is reasonable to conclude that the capacity of the collateral arterial bed to maintain a peak blood flow could account for the return of work performance to normal.

Blood flow in the vascular area subtended by the inflow system after total occlusion of the superficial femoral artery at the level used in this study fell to zero or near zero within seconds of occlusion and remained subnormal for 7 days. How much of the blood flow reduction at 7 days reflects the inadequacy of collateral arterial growth and how much reflects atrophy of disuse related to the injury is not clear. Sham-operated controls showed a similar blood flow level but may well have used their limbs more than did rats with femoral artery occlusion. Thus, the blood flow measured at 2–3 weeks after arterial occlusion reflects some combination of dilation and growth of the available collateral arterial tree. The results in the rat contrast sharply with our earlier findings in the dog,[11] in which at least a partial reconstitution of blood flow and angiographically evident collateral arteries were routinely observed within minutes of arterial occlusion. Presumably, the rat and the dog differ in the size of the available collateral arterial tree to the limb. Whatever the explanation, it is clear that the state of the collateral arterial blood supply 2–3 weeks after vascular occlusion is intermediate. The arteriograms revealed a far more extensive collateral

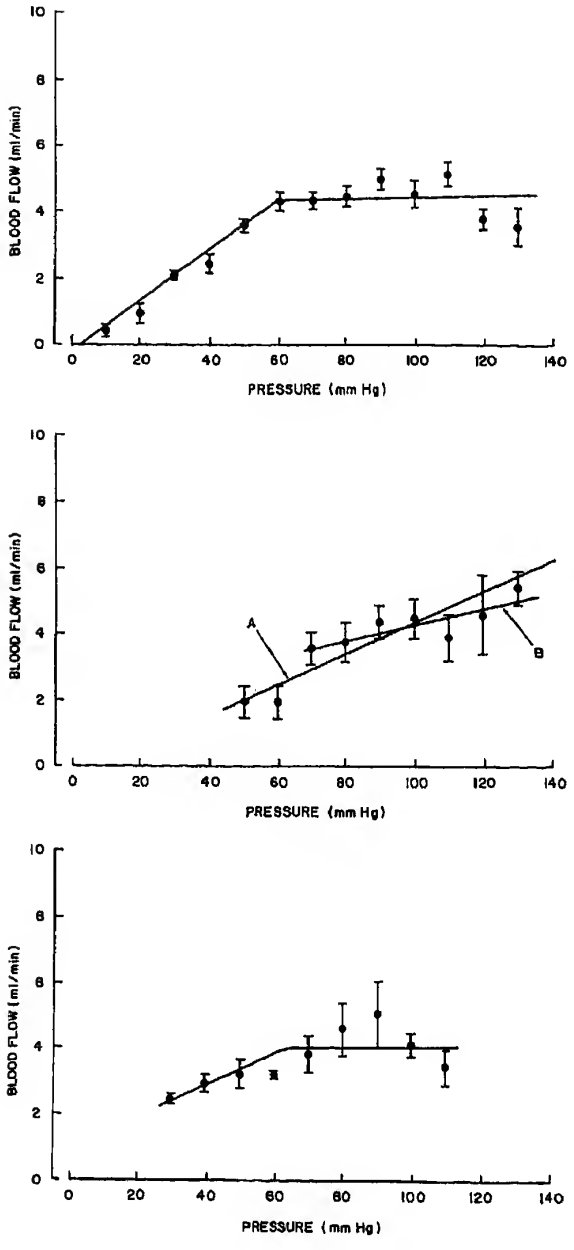

FIGURE 5. Top panel: *Pressure–flow relation in the femoral artery of the normal rat. Note complete autoregulation of blood flow from 60–120 mm Hg pressure in the steady state. Mean blood flow=4.4±0.18 ml/min; slope=0.* Middle panel: *At 2–3 weeks of collateral growth after femoral arterial occlusion, blood flow is a linear function of perfusion pressure over the pressure range of 50–130 mm Hg (line A). Mean blood flow=3.7±0.3833 ml/min; slope=0.0378±0.0069 (SD),* r=*0.90,* F=*30.2,* p<*0.01. Over the more limited pressure range of 70–130 mm Hg (line B), mean blood flow=4.2±0.23 ml/min; slope=0.023±0.007 (SD),* r=*0.82,* F=*10.2,* p<*0.001. The slope in each case differs significantly from 0.* Bottom panel: *The pressure–flow relation of collaterals at 3 months of development approaches that of normals. Autoregulation is reestablished at pressures greater than 60 mm Hg as the neovasculature matures. Over a pressure range of 60–110 mm Hg, the flat pressure–flow relation has been restored. Mean blood flow=4.03± 0.3 ml/min; slope=0,* r=*0.177,* F=*0.13.*

arterial tree at 3 months, and blood pressure–flow relations had returned to normal by 3 months.

We used a drop counting technique to assess blood flow to the rat limb. This method has a number of advantages and limitations. The advantages include great accuracy at very low flow rates, moment to moment measurement, and easy access to the control of perfusion pressure to the limb. The disadvantages include the need for anticoagulation, the need to isolate and open the femoral artery, and the possible local hemodynamic effects of placing a catheter into the very small femoral artery. The catheter used could have added a significant resistance to the flow through the limb. This seems unlikely in view of the close comparability of the pressure–flow relations for the limb identified in this study and described in many earlier reports.[16–19]

There have been a number of reports that suggest variation in the rate at which recovery of blood flow to the limb occurs after vascular occlusion. In the dog, a number of studies have documented a residual reduction in blood flow in the several weeks after femoral artery occlusion.[11,21–23] In the rat, a recent study claimed full recovery of blood flow, not only at rest but also during vasodilatation induced by muscle work.[12] None of the studies have reported pressure–flow relations, and indeed few describe measuring arterial blood pressure at the time of individual studies. It is conceivable that earlier, occasional reports of a normal blood flow in

the early weeks after femoral artery occlusion reflect experiments in which blood pressure was increased.

The recruitment of a collateral arterial supply appears to differ from tissue to tissue.[21,23] Although there appear to have been no reports on pressure–flow relations for the limb, there have been a substantial number of studies of pressure–flow relations during the growth of coronary collateral arteries.[24–27] Although the experimental protocols are not easy to compare, it appears that coronary collateral flow is more pressure dependent than perfusion of the limb, at least 2 or 3 weeks after femoral artery occlusion. Whether coronary collaterals reach a "mature" state in which pressure–flow relations are normal, as was documented 3 months after femoral artery occlusion in this study, has not been shown in any model.

Earlier reports have contrasted the characteristics of the collateral arterial supply to various vascular beds, and emphasis was given to differences between the collateral arterial supply to the heart and the limb.[3,21] The latter is thought to have a more immediately available collateral supply that effectively replaces the lost blood supply very quickly. The results of this study suggest that the differences may be more quantitative than qualitative. In the hour after acute occlusion, neither angiography nor direct blood flow measurement revealed an abundant available collateral arterial supply. Three weeks after femoral artery ligation in the rat, a substantial collateral arterial blood supply was evident, both by blood flow measurement and on the basis of anatomic examination by way of arteriography. On the other hand, studies at 3 months make it clear that substantial vascular growth occurs during the interval when followed, when the requirements of tissue need had been satisfied. The assessment of factors that modify vascular growth during that interval might provide insight into what is perhaps the most interesting question: What are the factors promoting vascular growth, and what is the communication system that mediates that process?

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